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4. Anonymous. Red cell aplasia [editorial]. Lancet 1982; 1:546-7.
5. Karlsson S, Humphries RK, Gluzman Y, Nienhuis AW. Transfer of genes into hemopoietic cells using recombinant DNA viruses [abstract]. Blood 1984; 64(Suppl 1):58a.

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6. Ferrata A, Storti E. Le malattie del sangue. 2nd ed. Milano: Vallardi; 1958.
7. Hillman RS, Finch CA. Red cell manual. 5th ed. Philadelphia: FA Davis; 1985.
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9. DuPont B. Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the third annual meeting of the International Society for Experimental Hematology. Houston: International Society for Experimental Hematology; 1974. p. 44-6.
10. Bieber MM, Kaplan HS. T-cell inhibitor in the sera of untreated patients with Hodgkin's disease [abstract]. Paper presented at the International Conference on Malignant Lymphoma Current Status and Prospects, Lugano, 1981:15.
11. Worwood M. Serum ferritin. In: Cook JD, ed. Iron. New York: Churchill Livingstone; 1980. p. 59-89. (Chanarin I, Beutler E, Brown EB, Jacobs A, eds. Methods in hematology; vol 1).
12. Ranofsky AL. Surgical operation in short-stay hospitals: United States-1975. Hyattsville, Maryland: National Center for Health Statistics; 1978. DHEW publication no. (PHS) 78-1785, (Vital and health statistics; series 13; no. 34).

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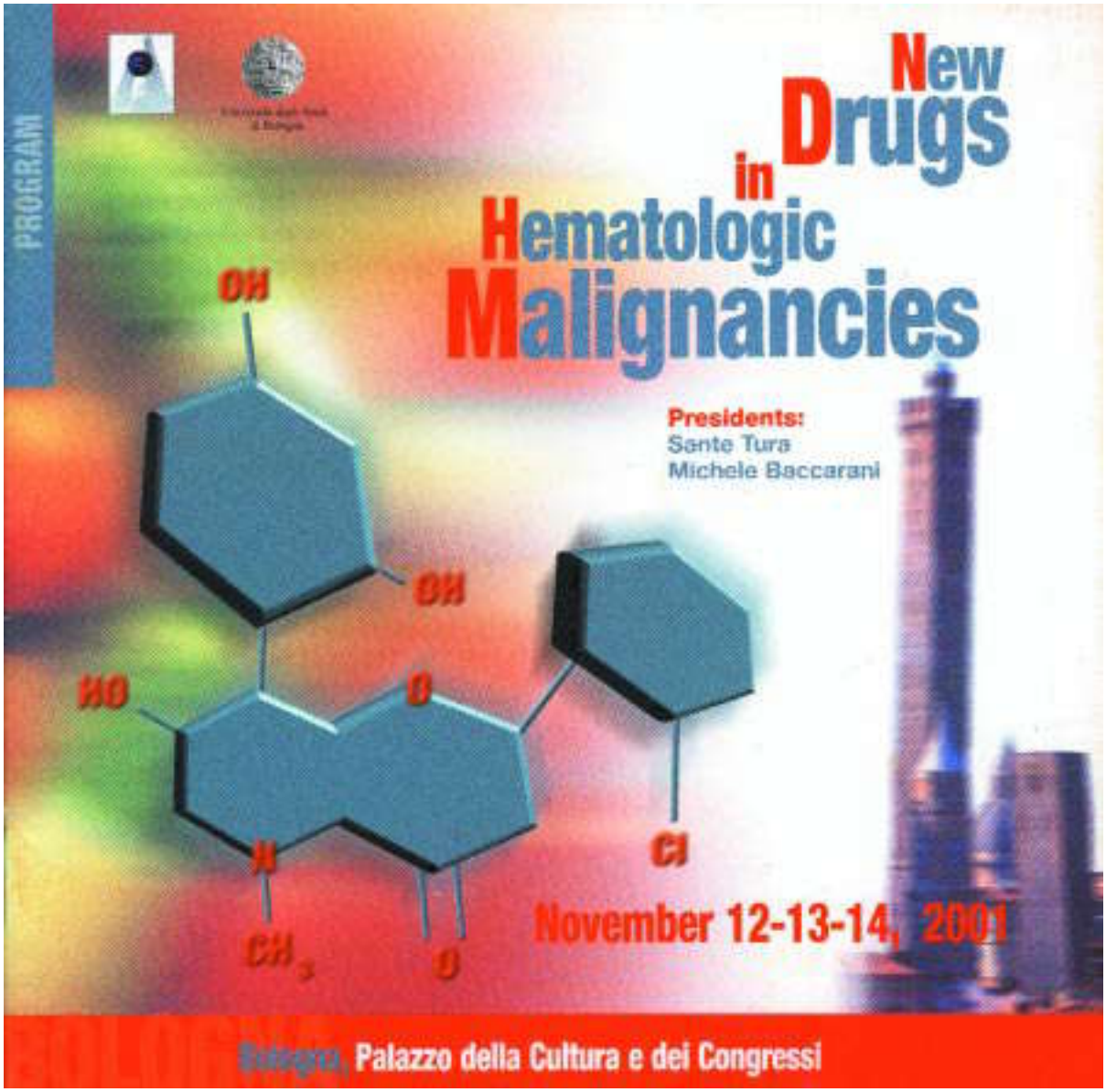
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Foreword

SANTE TURA
Institute of Hematology and Medical Oncology
"L. e A. Seràgnoli", Bologna University, Italy

In the last fifty years chemotherapy for hematologic malignant diseases has really improved. The percentage of cured acute leukemias is definitely comforting but, at the same time, urges us to reach new targets. Therapy for lymphomas cured a great number of patients. It was not drugs alone that helped us reach these goals but it was drugs above all.

We feel a sense of both curiosity and hope when new molecules enter clinical experimentation. Recently, this curiosity has increased remarkably because of the availability of drugs that hit the neoplastic cell directly limiting the involvement of normal cells. These are the so-called *intelligent* drugs on which we strongly rely.

The reason we decided to organize this Workshop was our need to talk about the assets and limits of the therapeutic action of those drugs that have already been experimented, of those still under experimentation and of those that will be experimented.

I hope this Workshop will be extremely productive and take this opportunity to thank all authors of this book because, reading it, we can think about the targets that we have already reached and those that we will reach in a short time.

Clinical Applications of Antiangiogenic Therapy (I)
Chairmen: T. Barbui, P. Calabresi

Angiogenesis in hematologic malignancies

ANGELO VACCA, ALDO M. ROCCARO, DOMENICO RIBATTI,* FRANCO DAMMACCO
Department of Biomedical Sciences and Human Oncology; *Institute of Human Anatomy, Histology and Embryology, University of Bari Medical School, Bari, Italy

New Drugs in Hematologic Malignancies
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Correspondence: Prof. Angelo Vacca, M.D. Department of Biomedical Sciences and Human Oncology, Section of Internal Medicine and Clinical Oncology. Policlinico, Piazza Giulio Cesare, 11. I-70124 Bari, Italy. Phone: international +39.080.5593106.
Fax international +39.080.5478820. E-mail: avacca@hotmail.com

Tumor angiogenesis is uncontrolled and unlimited in time, and essential for a tumor's growth, invasion and metastasis during the transition from the avascular to the vascular phase,[1] in which the tumor's proliferative activity is enhanced by new vessels that convey oxygen and nutrients and remove catabolites, while their endothelial cells secrete paracrine growth factors for tumor cells, such as insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (FGF-2). These vessels also facilitate both invasion, because endothelial cells at their sprout tips secrete proteolytic enzymes, such as metalloproteinases (MMPs) and plasminogen activators (PAs) that allow tumor cells to spread into and through the stroma, and metastasis, because the expanding endothelial surface offers tumor cells more opportunities to enter the circulation.

The vascular phase also parallels tumor progression in terms of neoplastic changeover.[1] The neoplastic or preneoplastic cell capable of inducing new vessels is ultimately responsible for the transition from the avascular to the vascular phase, e.g., for escape from the *dormant* phase.[1]

Tumor progression in multiple myeloma parallels the vascular phase in the bone marrow

Bone marrow biopsies of patients with active multiple myeloma (MM), non-active MM and monoclonal gammopathies of undetermined significance (MGUS) were investigated for microvessel density by using immunoperoxidase and an antibody to the endothelial cell marker factor VIII-related antigen.[2] Microvessels (e.g., capillaries and small venules) were selected and the area they occupied (*microvessel area*) was calculated per microscopic field by planimetric *point counting*. The average area of microvessels in patients with active MM was 5-6 fold greater than that of virtually similar microvessel collections in patients with non-active MM and MGUS. The area was closely correlated with the proliferative activity, as evaluated by plasma cell LI%,[2] and the widest area was seen in rapidly progressive disease. Overall, these findings suggest that, like solid tumor cells, highly proliferating plasma cells possess an angiogenic capability via the release of angiogenic cytokines. Since the progression from *in situ* to invasive and metastatic solid tumors is accompanied and enhanced by the switch from the avascular to the vascular phase, they suggest that active MM is the *vascular phase* of plasma cell tumors, and non-active MM and MGUS their *avascular phase*. Bone marrow angiogenesis may therefore favor progression from MGUS or non-active MM to active MM. Subsequent studies have confirmed substantial bone marrow neovascularization in MM.[3-7]

The vascular phase in other lymphoproliferative and myeloproliferative diseases

Excessive angiogenesis has been shown in lymph nodes of patients with hyperplastic and dysplastic B-cell disorders, such as Castleman's disease, related to or often associated with primary nodal plasmacytoma and osteosclerotic myeloma (the POEMS syndrome).[8]

As in active MM, enhanced bone marrow neovascularization and high urinary levels of FGF-2 have been shown in patients with acute lymphoblastic leukemia prior to treatment compared within a control group.[9] In other B-cell neoplasias, such as B-cell non-Hodgkin's lymphomas (B-NHL), we found that lymph node angiogenesis, evaluated as microvessel counts, was significantly enhanced

in relation with transition from low-grade to intermediate-grade (histotypes with diffuse growth pattern) and high-grade.[10-13] Thus, it is suggested that diffuse intermediate-grade and high-grade B-NHL correspond to the vascular phase, while low-grade and follicular intermediate grade B-NHL to the avascular phase. B-NHL-associated angiogenesis may be driven by mechanisms similar to those observed in MM: release of angiogenic cytokines directly by B-NHL cells and/or indirectly by the inflammatory infiltrate they recruit and activate.[14] We also found that the microvessel area expanded significantly with transition from the eczema to the plaque and nodule stage in mycosis fungoides.[15] Angiogenesis was paralleled by an increase in the proliferative activity of tumor cells.

These quantitative similarities between active MM, intermediate/high grade B-NHL and plaque-nodule mycosis fungoides are accompained by morphologic similarities in the form of very thin, winding, branching, mutually anastomosed microvessels and intact endothelial cell clusters resembling those of early tumor angiogenesis.[16]

Increased vessel density has been found in bone marrow specimens from patients with acute myeloid leukemia, implying that angiogenesis is a component of this latter's pathophysiology.[17] Similar conclusions have been drawn from studies of patients with myeloproliferative syndromes.[18,19]

Angiogenic cytokines involved in the progression of hematologic malignancies

We have obtained evidence in favor of FGF-2 production by myeloma plasma cells during the active phase.[20] Enzyme-linked immunosorbent assay (ELISA) on cell lysates of enriched plasma cells showed that FGF-2 levels were significantly higher in active MM patients compared with non-active MM and MGUS patients. These results agree with those obtained in patients with B-cell chronic lymphocytic leukemia, showing that overexpression of angiogenic interleukin (IL)-8[21] FGF-2[22] and VEGF[23] are correlated with progression. The FGF-2 secreted by myeloma plasma cells was operative, since a neutralizing anti-FGF-2 polyclonal antibody inhibited the angiogenic activity exerted on cultured human umbilical vein endothelial cells (HUVEC) by the plasma cell conditioned medium (CM). Inhibition was also obtained on angiogenesis induced in the chick embryo chorioallantoic membrane (CAM) by the CM.[20]

Bellamy *et al.*[24] observed expression of VEGF mRNA in 12 human leukemia, lymphoma and MM cell lines, and secretion of the corresponding protein into the extracellular environment. Five lines also expressed the Flt-1 (VEGFR-1) receptor at a moderate to strong level, suggesting an autocrine pathway of tumor growth. Plasma cells in the bone marrow of patients with MM expressed VEGF, whereas both Flt-1 and KDR (VEGFR-2) high affinity receptors were markedly elevated in the bone marrow stromal cells, suggesting a paracrine pathway (through IL-6) of tumor growth.

Hepatocyte growth factor/scatter factor (HGF/SF) is yet another angiogenic factor identified in the CM of both human myeloma cell lines[25] and freshly isolated myeloma cells.[26] Serum levels of this factor are high in 43% of patients at diagnosis, fall to normal levels if a response to induction therapy is achieved, rise again upon relapse.[27] Based on these observations and also on the fact that the corresponding levels fluctuate concordantly with the M-component serum/urine levels, HGF/SF has been interpreted as a typical secretion product of myeloma plasma cells. In patients with MM, FGF-2, VEGF and HGF/SF were all found to be more expressed in the bone marrow than peripheral blood plasma, indicating that the bone marrow is their prime source.[28]

Still other factors may be responsible for bone marrow angiogenesis in MM: i) the already demonstrated plasma cell secretion products,[29] such as transforming growth factor-β (TGF-β) and IL-1β; the latter factor, however, is only indirectly angiogenic insofar as it stimulates bone marrow microenvironment cells to secrete IL-6 and platelets to secrete PDGF (1); ii) IL-6, IL-8, G-CSF, GM-CSF, TNF-α,[29] secreted by bone marrow microenvironment cells recruited and activated by myeloma plasma cells (via chemokines? and, possibly, FGF-2). It is noteworthy that IL-6, IL-8, GM-CSF and IL-1β are secreted mostly in the MM active phase[30] during which we found enhanced angiogenesis.

Expression and secretion of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9)

We have used *in situ* hybridization to demonstrate that bone marrow plasma cells of patients with MM and MGUS express the mRNA for MMP-2 and MMP-9.[20] MMP-2 expression is usually stronger. Both MMPs are much more intensely expressed in patients with active MM than in those with non-active MM and MGUS. Their mRNA is operative, since zymography has revealed secretion of enzymes into the plasma cell CM.[31,32] In agreement with the hybridization results, MMP-2

levels in the CM were usually higher than those of MMP-9, and both MMPs were usually present in greater amounts in patients with active MM than in those with non-active disease and with MGUS. They were found in their cleaved (activated) form. MMP-2 and MMP-9 are 72-kDa and 92-kDa proteins, respectively, whereas their cleaved 62-kDa and 88-kDa forms were evident in the CM. MMPs may thus be rapidly cleaved as soon as they are secreted and activated by the membrane-type matrix metalloproteinase (MT-MMP) membrane system with which plasma cells could be equipped.[20]

Barillè *et al*.[33] have shown production of MMP-9 by an array of MM cell lines (prominently by the RPMI-8226 cell line) and freshly isolated bone marrow plasma cells of MM patients, though no correlation with disease activity was apparent. It has also been shown that bone marrow stromal cells (e.g., fibroblasts and osteoblasts) of MM patients constitutively produce MMP-1 and the MMP-2 proenzyme, which is converted into active MMP-2 in co-cultures with myeloma plasma cells.[33] Furthermore, IL-1β and TNF-α, i.e., cytokines produced by myeloma plasma cells and stromal cells, respectively,[29] enhance MMP-1 production. Still other studies describe IL-1β as a stimulator of the production of MMPs by bone marrow stromal cells.[34]

MMP-1 and MMP-2 are thus produced by bone marrow stromal cells stimulated by plasma cells (via IL-1β?). Given the ability of MMP-1 to degrade type I collagen and that of MMP-2 and MMP-9 to degrade type IV, V, VII and X collagens as well as fibronectin, i.e. the major components of the interstitial stroma and subendothelial basement membrane, the data suggest that plasma cells of active MM patients are especially capable of invading both the stroma and the basement membrane. In addition, degradation of extracellular matrix brings about the release of its stored angiogenic factors.[1] To sum up, overall data suggest that major proteolytic activity and angiogenesis occur together close to plasma cells and that the conditions for their intra- and extramedullary dissemination are established.[35,36]

Malignant B-cells in B-NHL are another substantial source of MMP-2, MMP-9[37] and urokinase-type PA.[38] The proteolytic activity connected with these enzymes is thought to be responsible for the extensive permeation of organs with destruction of their anatomical boundaries in a fashion similar to that observed in carcinomas.

Involvement of host inflammatory cells

In parallel with enhanced angiogenesis, we have obtained evidence on the increase of mast cell density in the bone marrow of patients with active MM.[39] At the ultrastructural level, most cells displayed the semilunar aspects of cytoplasmic granules, suggesting that they were chronically and slowly releasing mediators in response to a degranulatory stimulus. This study indicated that mast cells are recruited and activated in the bone marrow by more malignant plasma cells in active MM, and that angiogenesis is partly mediated by angiogenic factors (IL-6, IL-8, FGF-2, VEGF, GM-CSF) contained in their secretory granules. These results are in line with those demonstrating bone marrow angiogenesis in the SCID-hu MM model in close association with a dense stromal cell infiltrate.[40] In other B-cell malignancies, such as B-NHL, we found that as in MM mast cell[41] and macrophage[42] density increased with histologic progression, as defined both by Kiel and WF malignancy grades. Overall, these data agree with those showing a close relationship between mast cell/macrophage density and angiogenesis during progression of solid tumors.[43]

Possible prognostic value of angiogenesis in lymphoproliferative diseases

Multiple myeloma. The prognostic role of angiogenesis has already been demonstrated in patients with solid tumors, including breast, lung, colon, head and neck and prostate carcinoma: increased density of neovessels means poorer prognosis, because angiogenesis means a greater velocity of growth, and a greater invasive and metastatic capacity of the neoplastic cells. Hence, more vessels imply a locally more advanced and/or already metastatic disease.[44]

Bone marrow angiogenesis in patients with MM and MGUS is correlated with the proliferating activity of plasma cells, rated as LI%, itself a prognostic factor, since mean survival is shorter in patients with LI ≥1% (15 months) than in those with LI <1% (40 months), irrespective of the cell mass.[45] MM patients with high LI% respond rapidly to induction cytostatic treatment (*early responders*), but their response is short-lived and followed by an equally rapid relapse in connection with massive recruitment into the cell cycle of G_0 myeloma plasma cells, hence by shorter overall survival.[44] Since we have demonstrated that bone marrow angiogenesis and LI% are closely associated with the phases of MM activity and are mutually correlated, and since LI% is a prognostic factor, it may

well be that a given disease state (MGUS, non-active MM) is at risk of progression towards the subsequent larger-mass steady state (active MM) if the bone marrow shows angiogenesis. The risk would be the greater, the larger the microvessel area, namely it would be 3.9 times as great for each 1% LI increment. The risk is the same with 0.6% LI increments. Microvessel area variations are therefore less restricted than those of LI% as a risk assessment parameter, and could be a useful guide to prognosis, just as LI% is when applied in plasma cell proliferative diseases.[2]

B-cell non-Hodgkin's lymphomas. S-phase fractions <5% are typical of low-grade B-NHL, those >10% of the intermediate- and high-grade disease, and their prognostic value is similar to that of the histologic catagories, while the prognosis worsens in a given WF category when there are >6% increments in this fraction.[4,6] Our studies[10-13] suggest that diffuse intermediate-grade and high-grade B-NHL express an angiogenic phenotype which is absent in low-grade and follicular intermediate-grade subtypes. If the angiogenesis level in B-NHL were correlated with the S-phase fraction, as in MM, angiogenesis itself would acquire a similar prognostic value.

Conclusions

Overall, studies indicate that:

i) MGUS and non-active MM are avascular phases of plasma cell tumors, and active MM the vascular phase.
ii) Similar conclusions can be drawn from studies of low-grade/follicular intermediate grade and diffuse intermediate grade/high grade B-NHL, respectively.
iii) Tumor B-cells and T-cells induce the angiogenic switch via an array of angiogenic cytokines.
iv) Mast cells and macrophages in the tumor microenvironment are probably a source of angiogenic cytokines and participate in induction of the full angiogenic response.
v) Angiogenesis and secretion of proteinases may explain local and distant tumor dissemination.
vi) Angiogensis could be a prognostic marker for hematologic malignancies in much the same way as in solid tumors.

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Clinical Applications of Antiangiogenic Therapy (II)

Chairmen: T. Barbui, P. Calabresi

The meso-angioblast: a pluripotent, self-renewing cell that originates from the dorsal aorta and differentiates into mesodermal tissues

GIULIO COSSU
Stem Cell Research Institute, H. S. Raffaele, Milan and Dipartimento di Istologia ed Embriologia Medica, Universita' di Roma "La Sapienza", Rome, Italy

Correspondence: Giulio Cossu, Stem Cell Research Institute, H. S. Raffaele, Milan, Italy

The origin of post-natal, pluripotent *stem* cells is unknown; we proposed that they may be associated with the developing vasculature. To test this hypothesis we grafted quail or mouse embryonic aorta into host chick embryos. Donor cells, initially incorporated into the host vessels, were later integrated into mesodermal tissues, including blood, cartilage, bone, smooth, skeletal and cardiac muscle.

When expanded on a feeder layer of embryonic fibroblasts, the clonal progeny of a single cell from the dorsal aorta acquired an unlimited life-span, expressed hemo-angioblastic markers (CD34, Flk1 and c-Kit) at both early and late passages and maintained pluripotency in culture or when transplanted into a chick embryo.

We conclude that these newly identified, vessel-associated stem cells, the meso-angioblasts, participate in post-embryonic development of the mesoderm and likely represent the ancestors of post-natal stem cells.

Clinical Applications of Antiangiogenic Therapy (II)
Chairmen: T. Barbui, P. Calabresi

Antitumor activity of thalidomide in refractory multiple myeloma

PATRIZIA TOSI, ELENA ZAMAGNI, CLAUDIA CELLINI, DELIA CANGINI, MICHELE BACCARANI, SANTE TURA, MICHELE CAVO
Institute of Hematology and Medical Oncology
"L. e A. Seràgnoli", Bologna University, Italy

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Correspondence: Dott. Patrizia Tosi, Institute of Hematology and Medical Oncology "L. e A. Seràgnoli", Bologna University, Italy.

Multiple myeloma (MM) is a B-cell hematologic malignancy characterized by a progressive clinical course, usually within 3 to 5 years from diagnosis. Although novel regimens have been introduced through the years, results obtained with conventional chemotherapy have not significantly improved since the introduction of the melphalan + prednisone combination in the late sixties.[1] Recently, it has been demonstrated that more intensive chemotherapy with single or double autologous stem cell transplantation is superior to conventional chemotherapy as it produces longer disease-free and overall survival.[2,3] Allogeneic stem cell transplantation could further improve patient outcome, but it is applicable to a smaller percentage of patients and is associated with higher procedure-related morbidity.[4,5] It should also be pointed out that, unfortunately, a high percentage of patients relapse after autologous and even allogeneic stem cell transplantation, and re-treatment of these patients is sometimes very difficult due to poor marrow reserve and multi-organ toxicity. Novel therapeutic modalities are thus needed in order to find a cure for MM, and insights could derive from a better knowledge of the mechanisms that control cell growth and survival. It has recently been reported that angiogenesis plays a central role in MM progression,[6,7] as active disease is characterized by increased bone marrow neovascularization that is paralleled by increased angiogenetic potential of neoplastic plasma cells, mediated by secretion of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Inhibition of angiogenesis could thus be worth exploiting in MM therapy.

Thalidomide is a glutamic acid derivative that was initially developed for its sedative properties; after discovery of its teratogenic potential in the early sixties, its use was abandoned. Later on the drug was used in different settings, including cutaneous lepromatosis,[8] AIDS-related aphthous ulcers and wasting syndrome,[9,10] cutaneous lesions of systemic lupus erythematosus,[11] and cutaneous and pulmonary sarcoidosis.[12] The interest in the drug has also been renewed by demonstration of its activity in chronic graft-versus-host disease,[13,14] even though poor tolerance frequently limits drug usage in these patients.[15] Thalidomide has shown a broad spectrum of different effects; modulation of T-cell activity has been described, with induction of Th2 response and production of interleukin (IL)-2 and γ-interferon;[16] the compound also acts on bone marrow stromal cells causing inhibition of cytokine production, mainly tumor necrosis factor (TNF)-α[17] but also IL-6 and IL-12.[18] Thalidomide possesses a potent antiangiogenic activity in experimental systems;[19] it inhibits microvessel formation in the rat aortic ring assay[20] and it decreases vascular density in granulomatous tissue;[21] these effects are probably mediated by inhibition of bFGF and VEGF activity.[19,22] Based upon its antiangiogenic properties, thalidomide was used in solid tumors such as recurrent gliomas[23] and hormone refractory prostate cancer,[24] minor responses being observed in 2-30% of the cases. Clinical trials are presently ongoing on Kaposi's sarcomas and hematologic malignancies such as myelofibrosis[25] and chronic myeloid leukemia. Singhal *et al.*[26] were the first group to report on the efficacy of thalidomide as a salvage therapy in MM. Eighty-four patients with relapsed or refractory MM, most of whom had been previously submitted to autologous stem cell transplantation, were treated with thalidomide at

a dose of 200 mg/day, increasing the dose by 200 mg every two weeks, to a maximum of 800 mg/day. A > 25% decrease in serum or urinary paraprotein was observed in 32% of the patients, with two patients achieving a complete remission; the median time to response was 1.5 months and the median response duration was longer than 14.5 months. Since these data were reported, several groups have studied the effects of thalidomide in relapsed/refractory MM, obtaining comparable results[26-33] (Table 1). Side effects encountered during thalidomide therapy are mainly sedation, constipation and skin rashes; these effects are generally mild, but they can condition a patient's quality of life and lead to discontinuation of therapy, especially when high doses are administered.[34] Peripheral neuropathy, though rare, should not be overlooked, as it is a late-onset event thus occurring mainly in responding patients.[35] Toxic epidermal necrolysis, deep venous thrombosis and pulmonary embolism have also been reported in a lower percentage of cases.[35-37] Several issues concerning thalidomide therapy still need to be elucidated. First, it has not been clarified yet whether there is a dose-response effect, so what the optimal drug dosage should be is still a matter of debate. Barlogie *et al*.,[35] updating the results of the Arkansas trial, demonstrated that the cumulative dose at three months represents a predictor of response. Conversely, other groups[27,38] have shown that responses can be achieved at doses as low as 200 mg/day and even at 50 mg/day.[39] Second, the mechanism by which thalidomide exerts its action in MM is still unclear. After wide testing in relapsed/refractory patients, trials are presently ongoing using thalidomide, either alone or in combination, as first line therapy in MM. Rajikumar *et al*.[40] have shown a 38% response rate in untreated patients with smoldering MM, this being comparable to the rate obtained in pretreated patients. These results support the concept that thalidomide exerts its anti-

Table 1. Single agent thalidomide in relapsed/refractory multiple myeloma.

Author/Yr	*Nr of Pts*	*Dose/Day*	*% Response (> 25%)*
Singhal/1999	84	200→800	32
Weber/1999	46	200→800	50
Juliusson/2000	23	200→800	43
Kneller/2000	17	200→800	64
Hideshima/2000	44	200→800	39
Yakoub/2000	83	200→800	66
Pini/2000	5	200	70
Tosi/2001	11	200→800	72

neoplastic activity through pathways that are different from those followed by conventional antineoplastic therapy. Whether bone marrow angiogenesis is really perturbed by thalidomide therapy, however, is still under investigation. Early observations by Singhal *et al*.[26] showed no difference in bone marrow microvessel density (MVD) comparing bone marrow biopsies of sensitive and resistant patients. Conversely, Cheng *et al*.[41] demonstrated that an increased pre-treatment MVD could predict response to therapy. VEGF secretion by MM cell lines is not influenced by *in vitro* treatment with thalidomide according to Rajikumar *et al*.,[42] while Weber *et al.* demonstrated that VEGF serum levels were higher in responding patients[43] and results reported by our group[44] showed that the activity of thalidomide is superior in patients whose plasma cells secrete lower amounts of VEGF. Given these contrasting results, although inhibition of bone marrow angiogenesis has been claimed to be the major mechanism of thalidomide action in MM, other mechanisms could, presumably, be involved. Thalidomide is known to induce apoptosis of bone marrow plasma cells upon *in vivo* treatment[45] and

Table 2. Thalidomide + dexamethasone combinations.

Author/Yr	*Nr of Pts/Status*	*Thalidomide: dose/day*	*Dexamethasone: dose/schedule*	*% response*
Weber/2000	47/relapsed-refractory	200→800	20 mg/m²/d on d 1-5, 15-18	52 (> 50%)
Dimopoulos/2000	38/relapsed-refractory	200→400	40 mg/m²/d on d 1-4, 9-12, 17-20 then d 1-4/month	52 (> 50%)
Rajikumar/2000	26/untreated	200→800	40 mg/m²/d on d 1-4, 9-12, 17-20 odd courses; d 1-4/even courses	77 (>50%)
Palumbo/2000	37/relapsed-refractory	100	40 mg/m² on d 1-4/month	75 (> 25%)

to possess immunomodulatory activity, by stimulating T-cell proliferation and increasing NK-mediated cytotoxicity.[46] Indirect evidence supporting the co-operation of multiple pathways in thalidomide's activity could be the relatively rapid response (1-2 months) in sensitive patients.

Aiming at improving the results obtained with thalidomide as a single agent, several groups have tested the compound in combination with drugs that are known to be active in MM. Most clinical trials have been designed adding dexamethasone to thalidomide[40,47-49] (Table 2); the combination improved the efficacy of thalidomide and was also effective in patients who had previously been resistant to thalidomide or dexamethasone alone.[47] In newly diagnosed patients, oral thalidomide + dexamethasone produced a response in 77% of patients,[40] that is comparable, or even superior, to what can be obtained with VAD infusional chemotherapy. Other studies were conducted using thalidomide in combination with conventional chemotherapy; Moehler *et al.*[50] evaluated thalidomide plus cyclophosphamide-etoposide-dexamethasone (CED) in poor prognosis MM patients, obtaining 7% complete responses and 71% partial responses; Kropff *et al.*[51] used hyperfractionated cyclophosphamide in combination with thalidomide in relapsed-refractory MM patients, achieving 86% partial response; Munshi *et al.*[52] combined thalidomide with cyclophosphamide, etoposide, doxorubicin, cisplatin and dexamethasone (DT PACE) in patients with aggressive MM or plasma cell leukemia; a 40% response rate was obtained, with no adverse effects on subsequent stem cell collection.

The pleiotropic effects shown by thalidomide prompted investigators to search for compounds with more selective activity in order to understand the mechanism of action of thalidomide better and to identify drugs which could potentially target the disease with fewer side effects. Two classes of thalidomide analogs have been synthesized so far, the so-called *selected cytokine inhibitory drugs* (SelCIDs) which inhibit TNF-α production but have no effects on T-cells, and the *immunomodulatory drugs* (IMiDs), that stimulate T-cell activation and IL-2 and γ-IFN production.[31] These latter seem more promising as they have shown superior activity in reducing DNA synthesis and in inducing apoptosis in MM cell lines and in bone marrow plasma cells from MM patients. Phase I studies have shown responses even in thalidomide pre-treated patients.[53]

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Clinical Applications of Antiangiogenic Therapy (II)
Chairmen: T. Barbui, P. Calabresi

Antitumor activity of thalidomide in idiopathic myelofibrosis

Letizia Canepa, Filippo Ballerini, Riccardo Varaldo, Claudia Venturino, Marino Clavio, Ivana Pierri, Maurizio Miglino, Marco Gobbi
Clinica di Ematologia, DIMI, Università di Genova e Dipartimento di Ematologia e Oncologia, Azienda Ospedale S. Martino e Cliniche Universitarie Convenzionate, Genoa, Italy

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Correspondence: Prof. Marco Gobbi, Department of Internal Medicine, Division of Hematology, University of Genoa, Viale Benedetto XV 6, 16132 Genoa, Italy. Phone international +39.010.3538995. Fax: international +39.010.353.8953. E-mail: gobbi@unige.it

Myelofibrosis with myeloid metaplasia (MMM) is a chronic myeloproliferative disorder (MPD) characterized by the clonal proliferation of a myeloid stem cell, producing trilineage clonal expansion.[1] Lymphocyte compartment involvement has been reported to be heterogeneous.[2,3] The natural history of the disease is characterized at bone marrow level by an early cellular proliferative phase with minimal marrow fibrosis. Progressive functional marrow failure occurs as the progressive fibrotic phase develops, accompanied by ectopic and ineffective hematopoiesis, determining primarily spleen and liver enlargement. This later phase is often worsened by infections, hemorrhages, portal hypertension or transformation into acute non-lymphoblastic leukemia, which account for the high morbidity of the disease. Median survival ranges between approximately 3 and 5 years, with individuals reporting to have survived for as many as 30 years.[4]

Current therapy is only palliative. High dose chemotherapy followed by allogeneic bone marrow transplantation is potentially curative, but the high median age of patients at presentation prevents them from being eligible for this approach.[5] Traditional therapeutic options include: supportive transfusion of red blood cells and platelets; corticosteroids and androgens; cytostatics, particularly hydroxyurea; interferon-α. Anemia is often unresponsive to erythropoietin and splenectomy is indicated in selected cases, as perioperative morbidity and mortality are high in comparison with the risk of early and long term complications.[6] Occasional reports suggest the beneficial effect of immunosuppressive therapy.[7]

Several clinical and biological prognostic parameters have been studied with conflicting results. In 1996, Dupriez *et al.* proposed a simple scoring system based on two variables at diagnosis, hemoglobin (Hb) level and white blood cell (WBC) counts.[4] The authors emphasized that the scoring system may be improved by cytogenetics, but the insufficient number of patients with an evaluable karyotype at diagnosis precluded the selection of this parameter in multivariate analysis.

Current therapy does not improve survival and investigations of experimental alternative treatments are warranted.

Pathogenesis of myelofibrosis

The true clonal proliferative nature of MMM is confirmed by investigations involving X chromosome linked genes or their products,[1,2] cytogenetics[8] and ras mutation studies.[9] Nevertheless both marked neoangiogenesis and immunologic mechanisms supposedly play a role in the pathogenesis of the disease.

Bone marrow specimens of MMM patients show several histologic changes, particularly an increase of number of stromal cells, increased angiogenesis and modification of extracellular and intracellular levels of several fibrogenic, osteogenic and angiogenic cytokines.

The main pathogenetic mechanism of MMM involves the clonal proliferation of megakaryocytes and monocytes associated with an abnormal production and release of several cytokines. High levels of these cytokines (transforming growth factor β, basic fibroblast growth factor, platelet derived growth factor, vascular endothelial growth factor) induce polyclonal proliferation of fibroblasts and osteoblasts and, consequently, collagen fibrosis, new bone formation and increased bone marrow vascularity.[10]

In a recent retrospective study in a cohort of 114 MMM patients, Mesa *et al.* investigated the prognostic relevance of increased angiogenesis.[11] They concluded that marrow vascularity is significantly increased in MMM patients compared to in normal

controls and patients affected by other myeloproliferative disorders. Therefore increased angiogenesis, along with collagen fibrosis and osteosclerosis, may really be integral components of the bone marrow stromal reaction in MMM. Furthermore they demonstrated that only increased angiogenesis had an independent prognostic value, along with advanced age and percentage of circulating blasts, as a risk factor for survival. Increased marrow microvessel density was also associated with marked splenomegaly. As splenomegaly in MMM is a consequence of extramedullary hematopoiesis, a linkage is established between increased angiogenesis and ectopic (i.e. splenic) hematopoiesis; in other words increased angiogenesis favors the transition from the early (cellular) to the later phase of the disease (marrow fibrosis and ectopic splenic hematopoiesis). In conclusion angiogenesis is related to the progression of the disease. This report, therefore, provides rationale support for the therapeutic investigation of antiangiogenic agents in the treatment of MMM.

Thalidomide

Thalidomide was originally used in the 1950s in Europe to prevent morning sickness, but it was withdrawn from the market in the 1960s because of its teratogenic effects. The drug retains antiangiogenic properties[12] and also strong anticytokine activity. Thalidomide has been approved by the Food and Drug Administration for the treatment of cutaneous manifestations of erythema nodosum leprosum. Its potential therapeutic applications span a wide spectrum of diseases including infections, autoimmune and neoplastic disorders.

Recently some authors reported that neovascularization and angiogenic potential of plasma cells are related to progression of multiple myeloma (MM).[13,14] These findings provided the rationale for using thalidomide to treat MM patients refractory to conventional or high dose therapy.[15] The clinical response of a consistent fraction of refractory patients strengthens the suggestion that thalidomide might act through mechanisms alternative to those exploited by traditional agents used in MM. Studies are ongoing in order to clarify the mechanism of activity of thalidomide and its analogs against MM cells and significant results have already been reported.[16]

The above reported considerations about the pathogenesis of MMM coupled with the known antiangiogenic activity of thalidomide, represented the rational basis for investigating the therapeutic activity of thalidomide also in myelofibrosis. A preliminary study indicated that the drug might decrease transfusion needs and improve cytopenias.[17]

Clinical results

As far as we know only three extensive studies have been reported about use of thalidomide in MMM.[18–20]

In the group (21 patients) reported by Barosi *et al*.[18] a 6-month course of treatment was planned, but 19 patients (90.5%) discontinued the drug because of side effects. Only 13 patients received more than 30 days of therapy and were considered evaluable for a response. In these patients anemia improved in three out of seven (43%), thrombocytopenia in two out of three (66.%) and splenomegaly was reduced in four (30.8%).

In the six patients reported by Pozzato *et al*.[19] all completed 6 months of therapy (time of follow-up for all cases), with the well known side effects (asthenia, fluid retention and constipation). The three patients with *compensated* disease (not requiring transfusion) had a good response, with increased Hb levels, decreased spleen size and decreased WBC count. None of the *decompensated* patients showed any clinical or hematologic response.

Finally in the group of 10 patients reported by us,[20] three out of four patients affected by agnogenic MMM, treated within 1 year of their diagnosis, became transfusion independent, had a reduction of splenomegaly and an increase of platelet counts. They have now been receiving treatment for 9, 17 and 20 months (as of July 2001). The thalidomide dose has been tapered to the lowest dosage able to maintain the clinical response. With long term therapy neurologic side effects are persisting and sometimes worsening, as we noticed also in MM patients, even if the daily dosage is usually lower. Nevertheless no patients had to discontinue therapy. Among the six patients affected by secondary (post-polycythemia vera or post-essential thrombocytosis) myelofibrosis, no clinical response was observed. One patient developed progressive disease and one patient had to discontinue therapy because of confusion and depression.

Conclusions

Despite the low number of patients (37) some considerations might be drawn. In our opinion only patients with at least two months of therapy should be evaluated for response. In our three responding patients Hb levels > 10g/dL were reached after 2, 3 and 5 months of treatment and kept on rising even later; the same behavior was evident in Pozzato's series. In the series reported by Barosi the main side effects, which led to the discontinuation of therapy in 90.5% of patients within 3 months, were somnolence and depression. Constipation was also present in 47.6% of patients. Constipation and somnolence were present in a very high percentage of our patients

too, but WHO level was 1, so that no patient required discontinuation of therapy. We have noticed that constipation can be efficaciously prevented through medication with stool softeners or laxatives. As time went by the somnolence resolved spontaneously.

As outlined by Barosi, the open design of the studies and the nature of the compassionate protocol certainly require that encouragement and support are given to sustain compliance of the patients affected by these minor side effects. Perhaps the single center nature of the other two studies explains the high percentage of patients completing the planned treatment.

On the other hand the real emerging problem associated with long term therapy is related to worsening of neurologic side effects, demonstrated by both laboratory and clinical tests, because the reversibility of these symptoms is still under debate. We believe that this phenomenon is probably related to cumulative dose rather than to daily dosage.

In conclusion, the use of thalidomide in MMM seems to be efficacious and tolerable, even if biological studies are warranted to clarify the rationale of thalidomide therapy and clinical trials are required to choose the phase of the disease in which the drug might be more active (*compensated* patients? early phase of the disease?). Lower dose therapy might delay the appearance of neurologic side effects, which remain, in our opinion, the most important problem associated with this therapy.

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New Drugs
Chairmen: F. Mandelli, A. Pileri

Summary of the PEG-Intron experience in chronic myeloid leukemia

CRAIG TENDLER
Dept. of Oncology Research, Schering-Plough Research Institute, Kenilworth, NJ, USA

New Drugs in Hematologic Malignancies
Bologna, Italy
November 12-14, 2001

haematologica 2001; 86(suppl. I to n. 11):15-22

Correspondence: Craig Tendler, Dept. of Oncology Research, Schering-Plough Research Institute, Kenilworth, NJ 07033, USA.
E-mail: craig.tendler@spcorp.com

The purpose of this summary of results is to provide updated data from our phase I/II PEG-Intron Oncology program and the recent results from the pivotal phase III trial of PEG-Intron vs. INTRON A in subjects with newly diagnosed, chronic-phase chronic myeloid leukemia (C/I98-026). INTRON A is a well established, safe and efficacious treatment for chronic-phase chronic myeloid leukemia (CML); cytogenetic responses and long-term disease free remissions are achieved. Clinical experience to date suggests that treatment of CML with a long-acting interferon formulation could offer the patients important benefits, not the least of which is a significantly less taxing injection schedule and a possible improvement in compliance. Development of such an interferon formulation becomes especially important in light of the emerging role of oral tyrosine kinase inhibitors and the need to develop well-tolerated and potentially curative combination regimens with interferon for CML. An overview of the clinical studies included in the PEG-Intron Oncology development program is provided in Table 1.

Collectively, the phase I/II results demonstrate that PEG-Intron clearly has antileukemic activity, both as monotherapy and in combination with cytosine arabinoside (Ara-C). Furthermore, this efficacy is realized with good tolerability at dosages greater than 3.0 µg/kg/wk. These initial results, which have now been further confirmed in a pivotal phase III trial (C/I98-026), demonstrate the feasibility and clinical comparability of PEG-Intron weekly dosing to INTRON A, an approved standard of care for CML.

Clinical development program for CML

Phase I

One-month PEG-Intron monotherapy (C/I97-187). The objectives of this 4-week, single center, open-label, non-randomized, uncontrolled, rising multiple-dose study were determination of dose-limiting toxicity (DLT) and the maximum tolerated dose (MTD) of once weekly subcutaneous PEG-Intron, assessment of safety and tolerability, and pilot testing of a Health-Related Quality of Life (HQL) questionnaire in subjects with CML.

In general, the safety profile of PEG-Intron was similar to that which has been observed elsewhere for INTRON A. Dosages of PEG-Intron ≤9.0 µg/kg/wk were safe and well-tolerated as determined by both clinical and laboratory evaluations and assessments. All subjects experienced at least one adverse event. The most frequently reported adverse events were fever (23/27; 85%), headache (23/27; 85%), fatigue (20/27; 74%), rigors (20/27; 74%), and myalgia (17/27; 63%). There were no life-threatening adverse events, and the majority of events were mild in severity. There were 12 subjects who reported 29 severe adverse events that included: rigors (4 events), 3 events each of myalgia, fatigue and headache, fever (2 events), and 1 event each of hypotension, influenza-like symptoms, confusion, arthralgia, bone pain, sepsis, dyspnea, pneumonitis, acute renal failure, leg cramps, pain, pneumonia, urinary tract infection and cytomegalovirus infection. A dose-related increase in grade 1 to 2 (mild to moderate) toxicity was observed. There were no discontinuations or dose interruptions. A dose-related increase in grade 1 to 2 (mild to moderate) toxicity was observed. In the majority of subjects, white blood cell (WBC) counts remained stable or decreased during the 4-week treatment period, compared with prestudy levels. Most subjects exhibited a decrease in total WBC count during the 4-week study period. Serious adverse events, the majority of which were unrelated to PEG-Intron treatment, are presented in Table 2.

In this phase I study, PEG-Intron serum concentrations and area under the concentration vs. time

Table 1. PEG-Intron oncology studies (CML, renal cell carcinoma / other solid tumor, high-risk melanoma).

Clinical Phase, Study No. and Number of Subjects (n)	*Parameters Evaluated and/or Objectives of the Study*	*Study Design*	*Dosing Regimen Employed*
		CML	
Phase I, C97-187, n=27	Safety, tolerability and pharmacokinetics of PEG-Intron in previously treated subjects with CML	Four-week, rising, multiple-dose; non-randomized, open-label, uncontrolled study	PEG-Intron 0.75, 1.5, 3.0, 4.5, 6.0, 7.5 or 9.0 µg/kg/week SC
Phase I, C/I97-275 Combination PEG-Intron with Regimen A: n=23 Combination PEG-Intron with Regimen B: n=18 (total enrolled: n=41)	Safety and tolerability of PEG-Intron in combination with Ara-C and assessment of multiple-dose pharmacokinetics of PEG-Intron (all in previously treated subjects with CML	Four-week, rising, multiple-dose; non-randomized, open-label, uncontrolled study	Combination therapy: PEG-Intron 0.75, 1.5, 3.0, 4.5, 6.0, 7.5 or 9.0 µg/kg/week SC in combination with either Regimen A or Regimen B Regimen A: Ara-C 20 mg/m^2 10 days/month Regimen B: Ara-C 10 mg/day.
Phase I C/I97-235 PEG-Intron monotherapy, n=25[a] PEG + Ara-C, n=32[a] (total enrolled: n=57)	Primary Objective: Assessment of safety and tolerability of extended administration of PEG-Intron or PEG-Intron + Ara-C in subjects with CML who completed C97-187 (mono-) or C/197-275 (combination therapy). Secondary Objective: assessment of hematologic and cytogenetic responses in subjects with CML following administration of PEG-Intron or PEG-Intron + Ara-C	Length of treatment in study was individualized based upon subject response to therapy. Nonrandomized, open-label, uncontrolled, extension study for subjects who completed C97-187 or C/I97-275.	Extension study for subjects from C97-187 and C/I97-275. Individuals continued on with their prior dosing regimen. Thus C/I97-275 subjects continued with combination therapy and C97-187 subjects continued with monotherapy (both dosing regimens noted above).
Phase III; C/I98-026, n=344a	Efficacy and safety of PEG-Intron vs. INTRON A in subjects with CML	Treatment given for 48 weeks or until disease progression; multicenter, randomized, open-label, controlled study	Initial starting dosage regimen: PEG-Intron 6.0 µg/kg/week SC, or INTRON A 5 MIU/m^2/day SC. Dosage modification was permitted to assist in the management of treatment-related side effects.
		Renal Cell Carcinoma or Other Solid Tumor	
Phase I, C/I97-188, n=70a	Safety, tolerability and pharmacokinetics of PEG-Intron in subjects with solid tumors	Twelve-week, rising, multiple-dose; non-randomized, open-label, uncontrolled study	PEG-Intron 0.75, 1.5, 3.0, 4.5, 6.0, or 7.5 µg/kg/week SC
Phase I, C/I97-349, n=29a	Safety and tolerability of extended administration of PEG-Intron in subjects with solid tumors who completed C/I97-188	Forty-week, non-randomized, open-label, uncontrolled extension study for subjects who completed C/I97-188	PEG-Intron, 0.75, 1.5, 3.0, 4.5, or 6.0 µg/kg/week SC
		High-Risk Melanoma[b]	
Phase III; C/I98-135, n=126a	Efficacy and safety of PEG-Intron vs. INTRON A as adjuvant therapy for subjects with high-risk melanoma	Multicenter, randomized, open-label, controlled trial; duration of the study was 48 weeks for subjects who received INTRON A and 2 years for those who received PEG-Intron	PEG-Intron 6.0 µg/kg/wk SC x 2 years, or INTRON A 20 MIU/m^2 IV 5 days per week × 4 weeks followed by 10 MIU/m^2 SC TIW × 48 weeks

[a]closed to enrollment; study report pending. [b]closed for administrative reasons; safety and dosing analyses are ongoing.

curve (AUC) increased in a dose-dependent manner at week 1 but not at week 4. C_{max}, half-life values were not calculated due to the sparse sampling schedule. Although the PEG-Intron MTD was not reached in this short-term phase I trial, dosages of up to 9.0 µg/kg/wk were safely administered, and the toxicity profile was found to be qualitatively similar to that of Intron A.

One-month PEG-Intron and Ara-C (C/I97-275). C/I97-275 was a 4-week phase I study for previously treated individuals with CML. Subcutaneous PEG-Intron was administered weekly (0.75, 1.5, 3.0, 4.5, 6.0, 7.5 or 9.0 µg/kg/wk) in combination with one of two different fixed Ara-C dosage regimens: either Ara-C 20 mg/m^2 for 10 days per month or Ara-C 10 mg once daily each day throughout the month. The primary objectives were determination of dose-limiting toxicity (DLT) and maximum tolerated dose (MTD) of the study drug in this specific therapeutic environment and subject population. The two secondary objectives were the evaluation of the safety and tolerability of this agent in combination with Ara-C and the assessment of the multiple-dose pharmacokinetics of PEG-Intron in

Table 2. Serious adverse events with PEG-Intron: phase I study in subjects with CML (C/I97-187).

Subject Number (Age/Sex)a	*Dosing Duration*	*Adverse Event*	*Relationshipb to Study Drug*	*Outcome*
PEG-Intron 0.75 µg/kg/wk				
C97-187-01/001 (56/M)	1 day	Fever, skin infection (nos)	Not provided Unlikely	Hospitalized; continued treatment and completed the study
Same as above	3.5 weeks after study completion	Sepsis, acute renal failure, cytomegalovirus infection	Unlikely	Hospitalized and died due to sepsis, possibly secondary to cytomegalovirus disease
C97-187-01/002 (23/M)	2.5 weeks after study completion	Allogenic bone marrow transplant	Not provided	Hospitalized
PEG-Intron 6.0 µg/kg/wk				
C97-187-01/015 (59/F)	4 weeks after study completion	Increased hepatic enzymes	Probably related	Subject received 4 doses of PEG-Intron in the extension study (C97-235). Subject remained asymptomatic.
PEG-Intron 7.5 µg/kg/wk				
C97-187-01/018 (51/M)	3 weeks	Thrombocytopenia	Probably related	Lowest platelet count was 16,000; no transfusion given; completed the study
Same as above	22 days	Pneumonia	Unlikely	Hospitalized; completed the study
C97-187-01/021 (34/M)	3 weeks	Myalgia, speech disorder, cognition impaired, bone pain, spinal disorder	Unlikely	Hospitalized; herniated disc; completed the study

a:Sex: F=female, M=male; age is in years. b:Causality was determined by the investigator.

these subjects.

The study was not designed to evaluate the efficacy of either treatment, and therefore inferential analyses were neither planned nor performed. However, response to therapy was evaluated using standard definitions of hematologic and cytogenetic response, and patients who demonstrated improvement after 4 weeks of treatment were offered the opportunity to continue with therapy in an extension protocol (C/I97-235).

The criteria for evaluation of safety and tolerability were performance status, laboratory safety tests (serum chemistries, full blood counts and urinalysis), vital signs and blood samples for drug SCH 54031 concentration and serum neutralizing antibody (SNA) concentration.

The MTD was not reached and there was no DLT related to study drug administration that occurred during the study. Five serious adverse events occurred in 3 subjects. There was 1 discontinuation due to a severe but reversible adverse event of drug-related neutropenia following 2 weeks of dosing with 6.0 µg/kg/wk PEG-Intron plus Ara-C 10 mg daily. This subject was noted to have entered the study with mild myelosuppression at baseline.

A review of the most frequently reported treatment-emergent adverse events from subjects participating in the C/I97-275 study (including those adverse events that occurred during the extension study C/I97-235) is provided in Table 3. The most frequently reported treatment-emergent adverse events by severity are provided in Table 4.

Most adverse events were mild or moderate in nature and none was treatment limiting. One grade 4 event was reported; an episode of neutropenia in the 1.5 µg/kg/wk PEG-Intron/Ara-C 20 mg/m^2 group. When considering all the PEG-Intron plus Ara-C 20 mg/m^2 treatment cohorts, the most frequently reported (≥50% incidence) treatment-emergent adverse events were fatigue (83%), rigors (83%), fever (78%), headache (70%), nausea (65%), vomiting (61%), anorexia (52%), and increased sweating (52%). Overall, 22% (5/23) of these subjects reported grade 3 or 4 adverse events. When considering all of the PEG-Intron/Ara-C 10 mg daily treatment groups together, the most frequently reported (≥50% incidence) treatment-emergent adverse events were fatigue (78%), fever (78%), nausea (72%), rigors (72%), anorexia (67%), headache (61%), and diarrhea (56%). There were no reports of grade 4 adverse events in any of the PEG-Intron + Ara-C 10 mg daily dose groups.

Most of the observed changes in selected laboratory parameter values (hematologic and blood chemistry) in all of the PEG-Intron groups were CTC grade 1 or grade 2 from baseline values of 0 or 1. Hepatic enzyme grade shifts did not show a correlation with PEG-Intron dose; most were shifts from grade 0 to grade 1. No grade 3/4 hepatotoxicity was observed in any of the PEG-Intron + Ara-

Table 3. Most frequently reported treatment-emergent Adverse Events (all grades) by dosing cohort.

	Number of subjects (%) by PEG Intron dose cohort, µg/kg/wk (n=41[a])						
Adverse Event	*0.75 (n=6)*	*1.5 (n=6)*	*3.0 (n=6)*	*4.5 (n=5)*	*6.0 (n=7)*	*7.5 (n=6)*	*9.0 (n=5)*
Fatigue	6 (100)	6 (100)	5 (83)	5 (100)	6 (86)	5 (83)	5 (100)
Fever	5 (83)	5 (83)	4 (67)	5 (100)	6 (86)	5 (83)	5 (100)
Rigors	4 (67)	6 (100)	4 (67)	5 (100)	5 (71)	4 (67)	4 (80)
Nausea	2 (33)	6 (100)	4 (67)	5 (100)	5 (71)	4 (67)	3 (60)
Anorexia	4 (67)	5 (83)	4 (67)	4 (80)	4 (57)	3 (50)	5 (100)
Headache	3 (50)	5 (83)	5 (83)	4 (80)	5 (71)	3 (50)	3 (60)
Diarrhea	3 (50)	4 (67)	2 (33)	2 (40)	5 (71)	6 (100)	4 (80)
Sweating increased	2 (33)	5 (83)	4 (67)	3 (60)	6 (86)	2 (33)	3 (60)
Myalgia	2 (33)	3 (50)	2 (33)	2 (40)	7 (100)	3 (50)	2 (40)
Vomiting	0	3 (50)	2 (33)	4 (80)	3 (43)	2 (33)	3 (60)
Insomnia	3 (50)	3 (50)	2 (33)	1 (20)	2 (29)	3 (50)	3 (60)
Pain	1 (17)	2 (33)	1 (17)	3 (60)	4 (57)	3 (50)	3 (60)
Arthralgia	3 (50)	3 (50)	1 (17)	0	4 (57)	2 (33)	3 (60)
Dizziness	1 (17)	2 (33)	3 (50)	1 (20)	3 (43)	3 (50)	3 (60)
Depression	2 (33)	1 (17)	2 (33)	3 (60)	4 (57)	1 (17)	2 (40)

[a]*Includes data from C/I97-275 and C/I97-235.*

C dose groups. For other laboratory parameters, there was no increased frequency of grade shifts with increasing dose, for either regimen, over the course of the study.

The MTD was not reached. Adverse events were, in general, similar to those that have been observed and reported after the administration of INTRON A. There did not appear to be a correlation between the incidence or type of adverse event and the dose of PEG-Intron plus Ara-C. There was no increased frequency of laboratory grade shifts seen with increasing dose for either regimen over the course of the study. No evidence of serum neutralizing antibodies was found. Of the 41 PEG-Intron + Ara-C subjects in C/I97-275, 32 continued on into the extension protocol.

Table 4. Most frequently reported treatment-emergent Adverse Events by severity.

	Number (%) of Subjects (n=41)*	
Adverse Event	*All Grades*	*Grade 3 and 4*
Fatigue	38 (93)	8 (20)
Fever	35 (85)	–
Rigors	32 (78)	2 (5)
Nausea	29 (71)	1 (2)
Anorexia	29 (71)	–
Headache	28 (68)	3 (7)
Diarrhea	26 (63)	1 (2)
Sweating Increased	25 (61)	–
Myalgia	21 (51)	1 (2)
Vomiting	17 (41)	1 (2)
Insomnia	17 (41)	–
Pain	17 (41)	1 (2)
Arthralgia	16 (39)	–
Dizziness	16 (39)	–
Depression	15 (37)	2 (5)

**Includes data from C/I97-275 and C/I97-235.*

PEG-Intron or PEG-Intron Plus Ara-C in Previously Treated Subjects (Extension Study C/I97-235). C/I97-235 was an open-label, uncontrolled, extension study for subjects with CML who successfully completed their core protocols, studies C/I97-187 (PEG-Intron monotherapy) or C/I97-275 (PEG-Intron plus Ara-C). The primary objective was assessment of the safety and tolerability of extended administration of these respective treatments in this subject population. The secondary objective was assessment of hematologic and cytogenetic response following extended administration of these therapeutic regimens.

Individuals received the dosing regimens that they followed previously. Thus, subjects from C/I97-275 continued with PEG-Intron 0.75, 1.5, 3.0, 4.5, 6.0, 7.5, or 9.0 µg/kg subcutaneously each week in combination with either Ara-C 20 mg/m^2 for 10 days/month or Ara-C 10 mg daily (intermittent or daily therapy, respectively). Similarly, subjects from C97-187 continued with PEG-Intron monotherapy, 0.75, 1.5, 3.0, 4.5, 6.0, 7.5 or 9.0 µg/kg subcutaneously each week.

Dosage escalation of PEG-Intron was allowed to two dosage levels below the highest dosage currently being administered in the respective protocol. For example; if a subject was enrolled at a PEG-Intron monotherapy dosage of 0.75 µg/kg, and the

Table 5. Discontinuations from the extension protocol due to adverse events.

PEG-Intron Dosage, (µg/kg/week)	*Ara-C Regimen*	*Days on Treatment*	*Adverse Event(s)*
0.75	Intermittent	470	Anemia
1.5	Daily	148	Muscle atrophy and weakness, paresis, weight loss
3.0	Intermittent	38	Anxiety, depression
4.5	Intermittent	617	Erythema
6.0	Intermittent	150	Increased hepatic enzymes, increased alkaline phosphatase
6.0	Daily	72	Thrombocytopenia, depression, paresthesia, tremor
9.0	Intermittent	38	Bone pain, increased WBC count*

**Progression to blast crisis.*

current dosage in that protocol was 6.0 µg/kg, the subject may have had the dosage escalated to 3.0 µg/kg. Subjects receiving Ara-C in the previous study continued on the same dosage regimen of Ara-C. Ara-C administration was withheld if the WBC count fell below 2,000/µL or at the Investigator's discretion. Adverse event tabulations, ECOG performance, physical examinations, vital sign monitoring and clinical laboratory tests (full blood counts and serum chemistries) were recorded.

The duration of treatment in C/I97-235 was individualized based upon the subject's response to therapy. The criteria for partial hematologic response were WBC decrease of ≥50% from baseline, WBC ≤20,000/µL, or residual splenomegaly with a normal WBC count. Individuals were considered to have had a complete hematologic response with WBC <9,000/µL, platelets <450,000/µL and >100,000/µL, <2% immature forms in peripheral blood and no palpable spleen. A minor cytogenetic response was ≤90% Ph^+ cells while a major cytogenetic response was <35% Ph^+ cells.

At the end of two months of treatment, hematologic response was evaluated to determine eligibility for continuation in the study for an additional 3 months. Partial and complete hematologic responders were eligible to continue. After a total of 6 months of treatment, individual hematologic response was again evaluated to assess eligibility for an additional 6 months of therapy. Only complete hematologic responders were able to continue with treatment for a full 12 months. After this period, individuals achieving a minor cytogenetic response were eligible to continue treatment for an additional 12 months. After 24 months of therapy, subjects with partial or complete cytogenetic responses had the option to continue on study until disease progression.

The mean duration of treatment for C/I97-235 (n = 41, PEG-Intron plus Ara-C combination therapy) was 235 days (over 7 months); the range of days on treatment was 22 to 858. Subjects were able to stay on and tolerate therapy for substantial lengths of time, in some cases for over 2 years. In a small number of cases, under conditions of long-term administration of extension protocol therapy, fatigue, elevation of liver enzymes, hematologic toxicity and depression did become dose-limiting, resulting in study discontinuation in 7 cases (see Table 5). Only 5 individuals who discontinued treatment due to drug-related toxicity received less than 1 year of therapy. One subject treated with PEG-Intron 6.0 µg/kg/wk plus 10 mg/day Ara-C discontinued the core protocol (C/I97-275) after 2 weeks of treatment due to reversible neutropenia and thrombocytopenia, was able to continue on into this trial (C/I97-235), but then subsequently discontinued after 72 days. The incidence and timing of study discontinuations did not appear to be correlated with PEG-Intron dosage level. Table 5 summarizes adverse events that led to discontinuations from extended combination therapy.

The combination of PEG-Intron plus Ara-C (either the intermittent or continuous dosage schedule) was found to be active in this previously treated patient population with chronic-phase CML. Forty-six percent of subjects achieved a complete hema-

Table 6. Best hematologic and cytogenetic responses

Type of Response	*Number (%) of Subjects (n=41#)*
Hematologic*	
Complete	19 (46)
Partial	6 (15)
Cytogenetic°	
Major (complete plus partial)	9 (22)
Complete	6 (15)
Partial	3 (7)
Minor	7 (17)

**Five patients were unevaluable. °Six patients were unevaluable. #Includes data from C/I97-275 and C/I97-235.*

tologic response and 22% achieved a major cytogenetic response with the PEG-Intron and Ara-C combination regimen. Table 6 summarizes the best hematologic and cytogenetic responses.

Figure 1 summarizes the best hematologic and cytogenetic responses by dose cohort. A greater number of responses was seen in the higher PEG-Intron dosage groups (>3 µg/kg/wk).

Of the original 27 subjects from the core PEG-Intron monotherapy study (C97-187), 25 went on into the long-term extension study (C/I97-235). At the start of therapy 19 patients had active CML, and 8 patients were in complete hematologic response (CHR). Among the 19 patients treated, 7 (37%) achieved complete hematologic response; 2 (11%) had a complete cytogenetic response. Among the 8 patients in CHR, 7 (87%) improved cytogenetic response to complete. All 6 patients intolerant to interferon-α tolerated PEG-Intron; 4 improved their cytogenetic response. The results show that PEG-Intron is easier to deliver (once weekly), better tolerated, and perhaps more effective than interferon.

In this long-term extension study in previously treated subjects with chronic-phase CML, PEG-Intron, either alone or in combination with Ara-C, was demonstrated to have definite antileukemic activity. Furthermore, addition of Ara-C did not impede the deliverability of full therapeutic dosages of PEG-Intron. Based upon the responses seen in the higher PEG-Intron dosage groups and the tolerability data from the long-term extension study, the recommended dose for PEG-Intron for the phase III program was 6 µg/kg/wk.

Phase II/III

PEG-Intron vs. INTRON A in Newly Diagnosed Subjects (C/I98-026)

C/I98-026 was a randomized, controlled, multicenter, open-label phase III study of the efficacy and safety of PEG-Intron vs. INTRON A in adult subjects with newly diagnosed, chronic-phase CML. A total of 344 newly diagnosed, chronic-phase CML patients were randomized to receive either PEG-Intron at 6 µg/kg subcutaneously per week (n=171) or INTRON A 5 MIU/m^2 subcutaneously per day (n=173). The primary objective was to assess the efficacy of PEG-Intron vs. INTRON A; the secondary objectives were to assess the safety and impact on health-related quality of life (HQL) of PEG-Intron vs. INTRON A. The primary efficacy endpoint was major cytogenetic response (<35% Ph$^+$ cells) at 12 months. Subjects who discontinued study treatment prior to month 6 or did not achieve a complete hematologic response by that time were considered to be cytogenetic failures, regardless of their cytogenetic response at month 12.

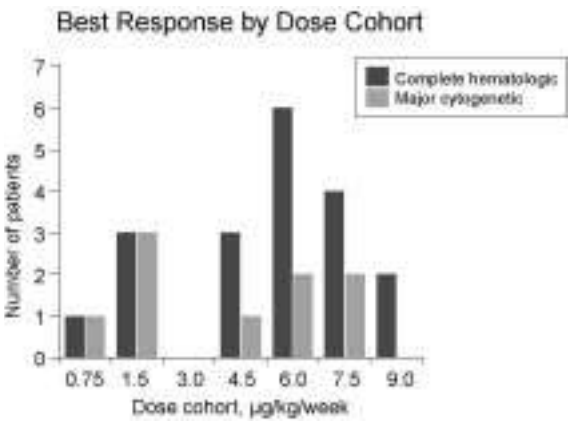

Figure 1. Hematologic and cytogenetic response by dose cohort.

Table 7. Summary of protocol-defined and sponsor-assessed efficacy for study C/I98-026.

Type of Efficacy Analysis	*Major Cytogenetic Response at 12 Months*		*Odds ratio*	*95% CI*
	PEG-Intron	*INTRON A*		
Primary efficacy	22.8%	27.7%	0.77	(0.47, 1.25)
Modified efficacy	26.3%	28.3%	0.91	(0.57, 1.45)

The secondary endpoints were hematologic response at 3, 6, and 12 months, cytogenetic response at six months, safety, HQL and population pharmacokinetics. A secondary, modified efficacy analysis, cytogenetic response at 12 months, defined prior to study unblinding, was also performed. This utilizes the same criteria as the protocol efficacy endpoint, but the modified assessment classifies, as responders, 7 cytogenetic responders who had borderline complete hematologic responses at six months, yet continued on study treatment. The modifed assessment also classifies five subjects who were month 12 cytogenetic responders as non-responders because they discontinued study treatment between months 6 and 12 and received other *effective* antileukemic therapy.

Safety and tolerability were assessed by clinical

Table 8. Subjects (n,%) With Most Common Treatment Emergent Adverse Events During Treatment Period (All Subjects) (C/I98-026).

A

B

C

observation and routine laboratory testing over the course of therapy. HQL was assessed by a validated questionnaire, the QLQc-30. Population pharmacokinetics was assessed by periodic serum sampling of the PEG-Intron group.

The median dosage delivered during the course of study treatment was approximately 5 µg/kg/wk for PEG-Intron (intended dose: 6 µg/kg/wk) as compared to 27 MIU/m²/wk for INTRON A (intended dose: 35 MIU/m²/wk). A treatment duration of at least 12 months was achieved by 67% and 68% of the PEG-Intron and INTRON A subjects, respectively. A summary of protocol-defined and sponsor-assessed efficacy at month 12 for both C/I98-026 treatment groups is presented in Table 7.

The complete cytogenetic response rates for PEG-Intron and INTRON A were 9.9% and 9.8%, respectively. The overall efficacy results, according to an independent panel of CML experts, are similar to those previously reported in the literature for interferon CML trials. The results demonstrate clinical comparability between PEG-Intron and INTRON A, although they do not quite meet the statistical criterion for non-inferiority (which requires the lower boundary of the 95% confidence interval to be >0.8).

In an effort to identify potentially confounding prognostic factors which may have an impact on cytogenetic response independently of treatment, a logistic regression analysis, as specified in the protocol, was performed. The three baseline patient variables which were highly significant (p<0.05)

Table 9. Subjects (n,%) with most common treatment emergent adverse events: grades 3 and 4 only during treatment period (all subjects) (C/I98-026).

for predicting major cytogenetic response to interferon therapy were hematocrit (patients with lower hematocrit had worse cytogenetic response), age, and the Hasford score (*Hasford J et al. JNCI 1998; 90:2580*). Notably, there was an imbalance in baseline hematocrit, the strongest predictor of cytogenetic response, between the treatment groups such that more patients with hematocrit <33 were randomized to receive PEG-Intron as compared to INTRON A (51 vs. 33).

A recursive partitioning analysis which utilizes all the baseline patient variables to identify homogeneous groups of patients most likely to respond to treatment also demonstrated that older subjects (>45 years of age) with hematocrit >32.7 were most likely to benefit from interferon therapy. In this uniform subgroup of patients (n=185) which was balanced for all other prognostic factors, the major cytogenetic response rate at month 12 was 40% for PEG-Intron as compared to 35% for INTRON A (odds ratio=1.22; 95% CI=0.67-2.23, relative risk=1.14; 95% CI=0.74-1.54).

Collectively, the supplemental analyses confirm the antileukemic comparability of PEG-Intron and INTRON-A and suggest that imbalance in a poor prognostic subgroup of patients with low hematocrit may have contributed to a reduced cytogenetic response rate in the PEG-Intron group.

PEG-Intron (6 µg/kg/wk subcutaneously) was safe and well-tolerated in subjects with chronic-phase CML. The adverse event profile for PEG-Intron and INTRON A was similar to that which has been reported previously in CML interferon trials (Tables 8 and 9).

Constitutional events, including subject discontinuations due to fatigue and asthenia, were reported in both groups. Depression, fatigue and headache more often resulted in study treatment discontinuation in the INTRON A group than in the PEG-Intron group. Injection site reactions were more commonly observed for PEG-Intron (42%) as compared to INTRON A (17%), but were usually of mild to moderate severity and rarely resulted in treatment discontinuation. Elevations in hepatic enzymes, and total bilirubin were within the range expected for this subject population. There were no reports of grade 4 hepatic laboratory abnormalities in either group, and grade 3 hepatic laboratory abnormalities were rarely reported. No subjects in the PEG-Intron group, as compared to 3 in the INTRON A group, discontinued for hepatotoxicity. No evidence of serum neutralizing antibodies was found.

The reported efficacy and safety results demonstrate that PEG-Intron is an active agent for the treatment of newly diagnosed CML patients, and that the weekly PEG-Intron dosage regimen has a similar safety profile as that reported for daily administration of INTRON A. PEG-Intron has the potential to provide an easier delivery schedule while maintaining similar efficacy and safety profiles with Intron A. Future studies will focus on evaluating PEG-Intron in combination with other active agents for CML, expanding the potential for safe and effective treatment options, and providing patients and clinicians with new therapeutic combinations that offer maximum flexibility, ease of administration, and improved patient acceptability.

New Drugs
Chairmen: F. Mandelli, A. Pileri

CMA-676 (gemtuzumab-ozogamicin; Mylotarg™)

Sergio Amadori, Roberto Stasi
Hematology, University Tor Vergata, Rome, Italy

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Correspondence: Sergio Amadori, MD, Hematology, University Tor Vergata, Rome.

Conventional treatment of acute myeloid leukemia (AML) consists of a combination of cytotoxic agents and results in complete remission rates of 60% to 80%. Unfortunately, similar proportions of patients who achieve remission eventually relapse, with a median survival time of approximately 18 months if not treated with stem cell transplantation. At present, less than 30% of AML patients survive more than 3 years.[1]

The availability of monoclonal antibodies (MoAbs) which react with antigens expressed only by hematopoietic cells has provided clinical investigators with new tools for developing innovative therapies for AML. Antibody therapy is ideally suited to treatment of hematologic malignancies because of the ready accessibility of malignant cells in the circulation. Furthermore, the observation that some MoAbs are internalized upon association with cell-surface antigens led to the development of antibody moieties that can selectively deliver toxins to targeted cells. The most promising of these novel drugs is CMA-676 (gemtuzumab ozogamicin), an antibody-targeted chemotherapy agent recently developed for the treatment of $CD33^+$ AML.

The CD33 antigen is a 67 kDa cell surface glycoprotein that functions as a sialic acid-dependent adhesion molecule. CD33 is expressed on the surface of leukemic cells in more than 90% of patients with AML.[2] This antigen is also expressed by myeloid progenitor cells and to a lesser degree by mature myeloid cells, but not by primitive hematopoietic stem cells or non-hematopoietic tissues.[3]

CMA-676 consists of a humanized IgG4-κ antibody (hP67.6) conjugated with calicheamicin, a potent antitumor antibiotic.[4] The antibody portion of CMA-676, which binds specifically to the CD33 antigen, is genetically engineered and contains murine light- and heavy- chain variable region sequences and human constant region sequences.

Mechanism of action

The binding of the anti-CD33 antibody portion of CMA-676 to the CD33 antigen results in the formation of a complex that is internalized. Upon internalization, the calicheamicin derivative is released inside the lysosomes of the target cell.[5] The released calicheamicin derivative binds to DNA in the minor groove resulting in DNA double strand breaks and cell death.[4] Calicheamicin can interfere with biological processes not simply by cleaving free DNA but also by displacing a DNA-binding protein complex through competition or modulation of the DNA structure.[6]

Clinical Studies

Preclinical results led to a phase I dose-escalation study of 40 relapsed or refractory AML patients.[7] The immunotoxin was generally well tolerated with the only consistent toxicities being the development of tolerable fever and chills several hours after the infusion of the drug, and the subsequent development of transient pancytopenia. Complete clearance of leukemic blasts from peripheral blasts and bone marrow was observed in 20% of patients, with the best response observed in those treated at the highest dose level (9 mg/m^2). Both safety and CD33 receptor site saturation data supported the choice of 9 mg/m^2, administered on days 1 and 15 by 2-hour intravenous infusion, as the appropriate dose-schedule for phase II studies.

Three open-label phase II studies (201, 202, 203) have been conducted in the United States, Canada, and Europe to evaluate CMA-676 further as a therapeutic agent in adults with $CD33^+$ AML in first relapse.[8] Studies 201 and 202 are nearly identical with the exception that study 202 was amended to allow the inclusion of patients with prior hematopoietic stem cell transplantation. Study 203 differs in some ways from studies 201

and 202 because the main objective in the study was to evaluate the effects of CMA-676 in older patients (≥ 60 years old).

The data from 142 patients show that the remission rate (defined as ≤ 5% blasts in the bone marrow, neutrophil count ≥ 1,500/μL and platelet transfusion independence) with CMA-676 was 32% in study 201, 33% in study 202, and 22% in study 203 for an overall remission (OR) rate of 30% (42/142). The lower remission rate for patients in study 203 was attributed to the older age and shorter duration of first remission of the patients in study 203. The median progression-free and the median overall survival were 9.5 and 5.9 months, respectively. The probability of survival beyond 3, 6, and 12 months was 73%, 50%, and 31%, respectively. The most common side effects were an infusion-related symptom complex and myelosuppression. The infusional syndrome was generally mild and consisted of chills, fever, nausea, hypotension and dyspnea. Myelosuppression was profound and universal, and the median time to ANC ≥ 500/μL was 42 days and to platelets ≥ 25,000/μL was 35 days from the first infusion of CMA-676. Liver function test abnormalities were common (32%), but were generally transient. There was no alopecia, a virtual absence of severe mucositis (4%) and low rates of severe infections (28%). The median duration of hospitalization was 24 days and the treatment-related mortality was 13%. All patients were tested for immune response, and none developed detectable antibodies against CMA-676.

Conclusions

The results of phase II trials suggest that single-agent CMA-676 administered to patients with AML in first relapse has an efficacy comparable with that of current salvage chemotherapy regimens as determined by a review of published literature and institutional databases of patients with similar key prognostic factors. The favorable safety profile supports its use in patients for whom intensive cytotoxic regimens would be considered unsuitable, such as many patients aged 60 years or more. Clinical trials are underway to explore the potential of this novel therapeutic agent further. One such trial (protocol AML-15/P) has recently been activated by the EORTC-LG in collaboration with GIMEMA, and involves elderly (> 61 years) patients with newly diagnosed primary or secondary AML. The design of the study is a dual treatment strategy based on risk assessment at diagnosis. Patients in the standard risk group (age 61-75 years and WHO performance status 0-1) are given CMA-676 as front-line therapy followed by standard induction chemotherapy (MICE regimen). On the other hand, poor risk patients (age > 75 years and performance status 0-2, or age < 76 years and performance status 2) receive CMA-676 as single agent for remission induction. This trial should therefore give important results regarding the safety profile of a combined program in standard patients, and explore the feasibility of CMA-676 alone in patients with a poor performance status. Whether the addition of CMA-676 confers a benefit to standard chemotherapy will remain to be determined in randomized phase III testing.

In conclusion, the treatment of AML is advancing rapidly and, like therapy in other malignant states, is favoring treatment strategies *tailored* to specific leukemia subtypes and patient categories. As more becomes known about the genetic and molecular characteristics of leukemia cells, it is hoped that future therapies will be directed specifically towards the clonal malignant cells, with the least amount of toxicity. At present, the use of a more specific anti-leukemic therapy such as CMA-676, may begin to change the disappointing outcomes now seen in elderly patients with AML.

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New Drugs
Chairmen: F. Mandelli, A. Pileri

Camptothecin and topotecan: state of the art

S. TURA,* M. MALAGOLA,* A. ISIDORI,* P.P. PICCALUGA,* G. VISANI°
*Institute of Hematology and Medical Oncology "L. e A. Seràgnoli", University of Bologna; °Department of Hematology and Transplant Center, H. San Salvatore, Pesaro, Italy

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Correspondence: Sante Tura, Institute of Hematology and Medical Oncology "L e A Seràgnoli", University of Bologna, Italy.

Camptothecin (CPT) is a plant alkaloid derived from *Camptotheca acuminata*. The first clinical studies reported severe and unpredictable toxic side effects, such as myelosuppression, diarrhea and hemorrhagic cystitis. Thus, clinical development of this agent was halted in the 1970s.[1-3] In the early 1980s, however, the molecular target of CPT, the topoisomerase-I (Topo-I) enzyme, was identified.[4] Subsequent investigations indicated overexpression of this Topo-I enzyme in various types of solid tumors, including ovarian and colonic cancer.[5,6] In addition, it was found that, due to poor water solubility, CPT necessitated a pharmaceutical formulation in alkaline solutions for i.v. administration, in this way reducing the previous shown toxicity.[7] New (semi-synthetic) analogs, such as topotecan and irinotecan were then identified and developed.

Chemical structure

Most of the currently known CPT analogs share a basic five-ring structure (Figure 1). The four lateral chains give different properties to the different CPT analogs, depending on the presence of alkylation, substitutions, hydroxy/methoxy radicals.

Mechanism of action

CPT and its analogs target the Topo-I enzyme, involved in scission and religation of DNA during replication and transcription phases. The primary mechanism of action of CPT is that of binding the Topo-I–DNA complex and interfering with the relegation step of this process, finally leading to double-strand DNA breaks and, ultimately, cell death.[8] Malignant cells often contain greater amounts of Topo-I than normal cells; they are frequently more sensitive to the toxic effects of CPT. The cytotoxic activity is specific to the S-phase of the cell cycle.

Pharmacokinetics and pharmacodynamics

CPT and its analogs are in a pH dependent equilibrium between a closed lactone E-ring and an open carboxylate form,[9] with a direct correlation between topotecan dose and plasma concentration peaks, as well as between topotecan dose and area under the plasma concentration/time curve (AUC) of lactone, especially if the drug is given in 24-hour intravenous infusions. The lactone ring is the active compound required for binding with the topo-I-DNA complex. However, at physiologic pH in human serum, the AUC of the lactone form is between 0 and 16% of the AUC of both forms combined. This may be the reason for the low therapeutic index observed from the start of topotecan infusion.[10] The half-life of topotecan is approximately three hours, and the drug has a large distribution volume, which suggests a high tissue uptake and low protein binding. Topotecan can pass through the blood-brain barrier. Excretion is mainly renal.

Clinical use of topotecan in solid tumors

The low activity, poor water solubility, and high toxicity of CPT have been successfully improved in semisynthetic derivatives, several of which are now being extensively studied in clinical trials in a wide variety of tumors including hematologic malignancies. Topotecan has been found to be effective in ovarian cancer resistant to cisplatin, with a response in about 30% of patients with relapsed or refractory disease. These results are similar to those obtained with paclitaxel in the same subset of patients. Unfortunately, the toxicity seems higher than that observed with paclitaxel, especially in terms of dose-limiting myelosuppression, even though this can be partially reduced with the use of granulocyte colony-stim-

ulating factor (G-CSF).[11] Other studies have shown a strong activity of topotecan in small cell lung cancer (SCLC), with responses in 30 and 29% of previously treated and untreated patients, respectively. Contradictory results have been obtained in non-SCLC.[12] Lynch *et al.* found that topotecan, at a dose of 2 mg/m^2, was not able to induce any response in 20 previously untreated patients with metastatic disease.[13] Rowinsky *et al.,* on the other hand, recorded 43% of responses in this latter subset of patients, using topotecan at a dose between 0.5 and 2.5 mg/m^2 for five days, whereas Verweij *et al.* observed 17% of responses using topotecan at a dose between 0.5 and 1.5 mg/m^2 for five days.[14,15] Finally, sporadic observations suggest topotecan activity in head and neck cancers, soft tissue sarcomas, gliomas and cervical carcinoma.

Topotecan as a single agent in hematologic malignancies

Acute leukemias. Topotecan as a single agent has been used in acute myeloid leukemia (AML). In two early trials the drug was administered as a continuous 5-day infusion. The dose-limiting toxicities were severe mucositis, diarrhea, mild nausea and vomiting. Myelosuppression-related fevers and infections were observed in about 75% of the patients. The maximum tolerated dose was found to be 10 mg/m^2 continuous i.v. infusion over 5 days (2 mg/day). In the first study responses (including both complete and partial remissions) were seen in 29% of the patients. The leukemic burden was reduced in all patients, whereas in the second study antileukemic effects of topotecan were observed, but no complete or partial responses. Topotecan as a single agent indubitably has activity in AML, and continuous infusion was selected as the route of administration for most of the subsequent studies.[16-18]

Concerning acute lymphoblastic leukemia (ALL), Uckun *et al.* reported on the activity of topotecan. A decrease in circulating and bone marrow blasts was noted in all patients treated with topotecan as a single agent, and the response was dose-related. However, the effects were only transient and disease progression generally occurred within 4 weeks. Other studies have suggested that topotecan as a single agent has a modest effect in untreated adult high risk ALL.[19]

Non-Hodgkin's lymphoma. A phase II study on 35 patients with indolent and 43 with aggressive refractory/relapsed non-Hodgkin's lymphoma (NHL) showed only partial responses to topotecan at a dose of 1.5 mg/m^2/day continuous infusion for 5 days, suggesting that topotecan has only a partial role to play in the treatment of lymphomas.[20]

Chronic lymphocytic leukemia. No significant responses and only modest and transient decreases in lymphocyte count were observed in a study on 12 patients with refractory chronic lymphocytic leukemia (CLL). The slow cycling populations of B-CLL cells is probably not sensitive enough to topotecan, even though high levels of the target enzyme Topo-I were found in B-CLL cells.[21]

Multiple myeloma. The efficacy of topotecan was tested in resistant or relapsing multiple myeloma, at a dose of 1.25 mg/m^2 for 5 days every 3 weeks in 30-minute i.v. infusions.[22] Although the response rate observed was low, any activity in therapy-resistant diseases, such as myeloma, is encouraging. The future lies in discovering whether or not higher doses of topotecan could be more effective.

Myelodysplastic syndromes and chronic myelomonocytic leukemia. The efficacy of topotecan has been tested in high-risk myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML). Topotecan was given at a dose of 10 mg/m^2, for 5 days, for two courses, as induction. Additional courses were given to responders, with appropriate dose-reduction. The CR rate was 30%, with a higher percentage of CRs in previously untreated patients. The response was more frequently achieved after the first course. After a median follow-up of 31 months, the median duration of CR was 7.5 months and the median survival was 10.5 months.[23-26]

Topotecan in combination with other therapeutic agents

One of the most commonly used combinations is topotecan plus AraC, in patients with MDS and CMML. This combination has been shown to produce an overall response rate of 56%, with a median CR duration of 50 weeks for MDS and 33 weeks for CMML. Besa *et al.* used the association of topotecan (dose escalation with a maximum of 3.0 mg/m^2/day for 4 days), fludarabine (30 mg/m^2/day for 4 days) and Ara-C (2 g/m^2/day for 4 days). G-CSF was added in order to reduce hematologic and extra-hematologic toxicity. Twenty-one patients with AML were treated (mean age 69.4 years). There were 2 deaths during induction, 10 CR, 8 PR and 1 progressive disease.[27] Leoni *et al.* have used a similar combination to treat elderly patients with AML. Dosages were, however, different: topotecan 1.25 mg/m^2/day for 4 days, Ara-C 2 g/m^2/day for 4 days, fludarabine 15 mg/m^2/day for 4 days. Twenty patients, with a mean age of 70 years; were

treated. Toxicity was not significant. The CR rate was 60%. After a median follow-up of 8 months, 10 patients (50%) maintained first CR.[18] Another valid combination seems to be the association of AraC (2 g/m^2/day for 5 days), topotecan (1.25 mg/m^2/day for 5 days) and cyclophosphamide (500 mg/m^2/12 hours for 3 days). With this combination Cortes *et al.* obtained 20% of overall responses (17% CR) in a subset of 52 patients with refractory/relapsed AML and 11 with refractory/relapsed ALL (median age 57 years [18-79]).[28]

This therapeutic schedule seems to be better tolerated than other therapies used for this subset of patients and the results were comparable to those obtained with FLAG or high-dose AraC chemotherapy. The association seems to be particularly efficient in patients with karyotypic alterations associated with a poor prognosis. How to maintain remission is a major problem. One possibility is to use such a well-tolerated therapy in selected young patients to induce complete remission and to reduce tumor burden before allogeneic bone marrow transplantation. The best therapy for this subset of high-risk AML patients still has to be defined.

Regarding MDS, Beran *et al.* have recently reviewed the data concerning the combination of topotecan and intermediate or high dose Ara-C with or without the addition of cyclophosphamide. The CR percentages were 59 and 58% in patients treated with topotecan plus intermediate or high dose AraC, respectively. The survival was longer in the former of the two groups of patients.[29]

Combinations with other cytotoxic drugs, such as cisplatinum, have also been tested. The rationale for these associations is the enhanced cytotoxic effects of topotecan when associated with DNA-damaging agents. In particular, combining topotecan with inhibitors of topoisomerase II, such as etoposide, secus, at least *in vitro*, resulted in a synergism as after administration of topotecan there is downregulation of Topo-I and up-regulation of Topo-II. Based on this, Nand *et al.* treated 11 patients with refractory AML (mean age 63 years) with mitoxantrone (6 mg/m^2/day for 5 days), etoposide 80 mg/m^2/day for 5 days) and topotecan (1.5 mg/m^2/day for 3 days). The CR rate was 33%. Unfortunately, CR duration was short (between 2 and 7 months).[30]

Different doses have, however, resulted in better response: in fact, Hoechsmann *et al.* obtained 68% CR after one cycle of topotecan (1.5 mg/m^2/day for 5 days), AraC (1 g/m^2/day for 5 days) and mitoxantrone (12 mg/m^2/day for 2 days) in 30 patients with refractory/relapsed AML (median age 59 years). The CR rate rose to 85% after a second cycle.[31]

In conclusion, topotecan appears to be an interesting drug, in particular considering its mild toxicity, for frail subjects such as elderly (AML or MDS) patients. A better definition of adequate combinations with other drugs, as well as of proper consolidation regimens after achieving CR is still necessary.

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New Drugs

Chairmen: F. Mandelli, A. Pileri

Zoledronate

Michele Cavo, Claudia Cellini, Elena Zamagni, Delia Cangini
Institute of Hematology and Medical Oncology
"L. e A. Seràgnoli", Bologna University, Italy

Correspondence: Michele Cavo, Institute of Hematology and Medical Oncology "L e A Seràgnoli", University of Bologna, Italy.

Zoledronate is a new-generation, nitrogen-containing bisphosphonate that has been shown to be 40- to 850-fold more potent than pamidronate in preclinical models of bone resorption.[1] These premises provided the rationale for several phase II-III clinical trials which were conducted over the last few years and were aimed to prospectively compare zoledronate vs. pamidronate in the treatment of both hypercalcemia and bone disease associated with malignancies.

In the first of these trials, 285 patients with hypercalcemia, as defined by corrected serum calcium levels ≥ 12 mg/dL, were randomized to receive a single dose of zoledronate, 4 or 8 mg in a 5-minute infusion, or pamidronate, 90 mg in a 2-hour infusion.[2] Efficacy variables included rate of complete response (CR) by day 10, response duration and time to relapse. Both doses (4 mg and 8 mg) of zoledronate were superior to pamidronate in terms of CR rate (88.4% and 86.7% vs 69.7%, respectively), CR duration (32 and 43 vs 18 days, respectively) and time to relapse (30 vs 40 days, respectively), with an advantage favoring 8 mg zoledronate as for CR rate by day 4 and duration of CR.

In another trial, zoledronate given at 3 different doses (0.4 mg, 2.0 mg or 4.0 mg, via 5-minute infusion) was compared to pamidronate, 90 mg in a 2-hour infusion, in the treatment of bone disease due to breast cancer or multiple myeloma.[3] Two-hundred eighty patients entered this phase II, dose-finding study and were evaluated for both the need of radiation therapy to bone and the frequency and type of skeletal-related events within the 10-month treatment period. The need for radiation therapy in the 2.0 mg and 4.0 mg zoledronate groups and in the pamidronate group was 19%, 21% and 18%, respectively. These values, but not the 24% rate observed in the 0.4 mg zoledronate group, were significantly lower than that expected (e.g. 30%) in a similar population of patients treated with antineoplastic therapy without bisphosphonates. Skeletal-related events of any type, pathologic fractures and hypercalcemia also occurred less frequently in patients treated with 2.0 mg or 4.0 mg zoledronate or pamidronate than in patients receiving 0.4 mg zoledronate, with a slight advantage favoring 4.0 mg zoledronate as for the probability of developing hypercalcemia and time to first skeletal-related event.

Based on these data, zoledronate doses of 4.0 mg and 8.0 mg were subsequently explored in a large, multicenter trial which was designed to compare the efficacy and safety of zoledronate with those of standard-dose pamidronate in the treatment of bone disease associated with multiple myeloma or breast cancer.[4] A total of 1,648 patients entered this study and were stratified according to their malignancy and type of therapy for breast cancer (hormonal or chemotherapy). Zoledronate was initially infused over 5 minutes in 50 mL of hydration solution; due to concerns over renal safety, a protocol amendment subsequently changed the infusion time to 15 minutes and increased the volume of hydration solution to 100 mL. An additional amendment to the protocol resulted from concerns over renal safety at the higher zoledronate dose of 8.0 mg and required a dose reduction to 4.0 mg. No difference between treatment groups was observed with respect to the primary efficacy variable which included the proportion of patients experiencing at least one skeletal-related event (excluding hypercalcemia) over the 12-month treatment period of the study.

More specifically, the observed rates in the 4.0 mg and 8.0 mg zoledronate groups and in the pamidronate group were 44%, 46% and 46%, respectively, with no difference among breast cancer and multiple myeloma patients. Rates of hypercalcemia, pathologic fractures, vertebral fractures, spinal cord compression and surgery to bone were also similar between treatment groups. However, compared to pamidronate, 4.0 mg zoledronate provided a significantly greater clinical benefit with respect to the incidence, event rate and time for radiation therapy to bone (20% vs. 15% and 0.71 vs. 0.47 events/year, respectively) and also significantly reduced the urinary excretion of N-telopeptide, a valuable marker of bone loss.

The safety profile of 4 mg zoledronate, given in a 15-minute infusion, was similar to that of pamidronate, particularly with respect to renal tolerability. Indeed, changes from normal baseline serum creatinine values were oberved in 9% of patients treated with 4.0 mg zoledronate and in 8% of patients receiving pamidronate, whereas only 1% of patients in both treatment groups had a grade 3-4 increase in serum creatinine. In conclusion, results of these studies showed that 4.0 mg zoledronate, in a 15-minute infusion, was superior to pamidronate in the treatment of hypercalcemia of malignancy, while having a similar activity in reducing skeletal-related events associated with multiple myeloma or breast cancer. Finally, the 15-minute infusion rate of zoledronate may represent an advantage over pamidronate, which must be administered in an infusion lasting 2 hours.

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Next New Drugs
Chairman: R. Danesi

Next new drugs: ...to the phase II trials

BRUCE D. CHESON
National Cancer Institute, Bethesda, MD, USA

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Correspondence: Bruce D. Cheson, M.D., National Cancer Institute, Bethesda, MD, USA

Novel strategies are needed to improve the prognosis of patients with hematologic malignancies. One approach is to identify new drugs with unique mechanisms of action. Nucleoside analogs, such as fludarabine, have become standard therapy for chronic lymphocytic leukemia (CLL) and the indolent non-Hodgkin's lymphomas (NHL). Nucleoside analogs in development include clofarabine (2-chloro,-2'-fluoro-arabinosyladenine) which has shown activity in patients with CLL and prolymphocytic leukemia (PLL),[1] troxacitabine,[2] and bendamustine, a hybrid of an alkylating nitrogen mustard group and a purine-like benzimidazol, with demonstrated activity in CLL, NHL and myeloma.[3] Giles *et al.*[2] recently published the results of the first phase I trial in 42 patients with acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML). The dose limiting toxicities were cutaneous, mucositis and hand foot syndrome. There were 4 complete responses (CR) in patients with AML (13%), 1 hematologic improvement in a patient with MDS, and a CR in a patients with CML in blast crisis. Bone marrow hypoplasia occurred in 71% of the AML patients. Combinations are planned with cytarabine, idarubicin, topotecan, and Mylotarg.

Interest in arsenic trioxide as an anticancer agent was stimulated by its demonstrated efficacy in acute promyelocytic leukemia (APL).[4] Sensitivity to arsenic trioxide *in vitro* has also been demonstrated against a variety of tumor types including myeloid leukemias, myeloma, lymphoid leukemia and lymphoma and various solid tumors. Arsenic induces apoptosis of malignant lymphocytes at clinically achievable doses. Arsenic reduces survival and viability of both NHL and CLL cells with a significant decrease in bcl-2 expression and apoptosis of CLL cells.[5,6]

DNA hypomethylation and histone deacetylation

Recent developments in understanding the molecular basis of transcriptional repression and activation have presented new possibilities for cancer therapy. Two mechanisms of gene silencing, promoter hypermethylation and histone deacetylation, appear to be interrelated. The process of neoplastic progression is linked to imbalances in DNA methylation. Hypermethylation of gene promoter regions is associated with repression of genes that regulate tumor growth and differentiation. Histone acetylation modulates higher order chromatin structure. Acetylation of lysine tails leads to loosening of DNA-histone contacts, which results in increased accessibility of transcription factors and increased gene expression. Co-activator histone acetylase complexes promote gene expression whereas co-repressor histone deacetylase complexes inhibit gene expression. Examples of the role of histone deacetylase in leukemia have been reported in the setting of PML-RAR[7,8] and PLZF-RAR[9] as well as with AML-1/ETO.[10-12] There is potential synergy between DNA methylation inhibition and histone deacetylase inhibition in restoring gene expression silenced by hypermethylation.

Agents that inhibit histone deacetylase *in vitro* include sodium phenylbutyrate (PB), depsipeptide, and hybrid polar compounds.[13,14] These agents induce terminal differentiation *in vitro* as well as cell cycle arrest and reversion of the malignant phenotype of a variety of malignancies including myeloid leukemias.[15-20] PB may have synergistic activity with ATRA in the treatment of APL.[21-24]

Depsipeptide (NSC 630176) is a bicyclic peptide originally isolated from *Chromobacterium violaceum*, strain 968, by Fujisawa Pharmaceutical Co., Ltd., that has been shown to be a histone deacetylase inhibitor.[25] Incubation of chronic lymphocytic leukemia cells with depsipeptide resulted in an alteration in apoptosis-associated pro-

teins: an increase in BAX with no change in Bcl-2, and a decrease in p27 expression.[26] Impressive activity has been noted in patients with T-cell lymphomas, particularly cutaneous T-cell NHL.

5-azacytidine and 5-aza-2-deoxycytidine are hypomethylating agents that are metabolized intracellularly to triphosphates and subsequently incorporated into newly synthesized DNA where they directly inhibit DNA synthesis and inhibit the activity of DNA methyltransferase, the enzyme required for 5'-cytosine methylation of CpG dinucleotides. As a result, cytosine methylation is blocked in newly replicated DNA, but not in the DNA of resting or non-dividing cells. Inhibition of methylation by 5-azacytidine and decitabine is associated with transcription of genes previously silenced by methylation of promoter region CpG-rich islands, and with cellular phenotypic changes; these effects can occur at concentrations that are too low to inhibit DNA synthesis directly or cause substantial cytotoxicity.

The combined administration of a demethylating agent and a histone deacetylase inhibitor has been shown to have synergism in reactivating genes that were silenced in cancer cells.[27] Such combinations represent a unique strategy that is currently being studied in patients with AML and MDS.

Antitubulin agents include dolastatin-10, a naturally occurring pentapeptide isolated from the marine mollusk *Dolabella auricularia*. It binds tubulin and inhibits microtubule assembly and tubulin-dependent GTP binding, and causes cell arrest.[28] The level of intracellular accumulation correlates with cytotoxic potency. Dolastatin-10 is one of the most potent *in vitro* cytotoxic anticancer compounds. The compound has antitumor activity against human leukemia and lymphoma cell lines and solid tumors in a variety of *in vitro* assays. Cytotoxic effects of the compound may be related to modulation of apoptosis-associated proteins, such as bcl-2. Reversible myelosuppression was the dose-limiting toxicity in phase I trials. Epothilone B analogs appear to be effective even in taxane-resistant cells and anecdotal responses have been reported in lymphomas.[29]

Flavopiridol is a semisynthetic flavone derivative derived from the plant alkaloid rohitukine isolated from the leaves and stems of *Dysoxylum binectariferum* used in India as a herbal medicine. Flavopiridol has *in vitro* activity against both cycling and non-cycling cells. Its antitumor activity may reflect the inhibition of cyclin-dependent kinases (CDKs), which regulate progression through the cell cycle. These include cyclin D1 which has been implicated in the pathogenesis of mantle cell lymphoma (MCL), as well as CDK1, CDK23, and CDK4. It induces growth arrest, cytotoxic cell death and apoptotic changes in a variety of tumor types, including leukemias and lymphomas. It has also demonstrated sequence specific synergy with cell cycle active agents including fludarabine and cytarabine. Flavopiridol induces apoptosis of B-CLL and lymphoma cells. Little activity has been observed using a 72-hour infusions schedule, whereas responses have been reported with a one hour schedule in CLL and MCL.[30-34]

UCN-01 is an analog of staurosporine isolated from a Streptomyces species originally identified as a selective inhibitor of PKC.[35-37] Subsequently, it has been found to inhibit a number of serine/threonine kinases with arrest of cells in G1 and abrogation of the G2/M checkpoint. UCN-01 demonstrated cytotoxic effects *in vitro* and *in vivo* against a variety of murine and human malignant cell lines. UCN-01 can induce apoptosis in leukemic cells lacking functional p53 and resistant to apoptosis induced by DNA-damaging agents. Dose-limiting effects include hypoxia, self-limited hyperglycemia, lactic acidosis with hyperglycemia, nausea and vomiting and transient elevation of liver transaminases. UCN-01 appears to potentiate the activity of fludarabine[38] and a combination of the two drugs in CLL is being studied at the NCI.

The family of Ras genes encodes 21-kd proteins which function as molecular switches that regulate diverse signaling pathways involved in cell growth, differentiation and apoptosis. The first step in the process involves the enzyme farnesyl transferase. Therefore, farnesyl transferase inhibitors (FTIs) are of interest. FTIs in clinical development include R115777 (Janssen Pharmaceuticals), BMS-214662 (Bristol-Meyers-Squibb), and LB-42908 (LG Chem). Activity was seen in AML and MDS in phase I trials.[39] An increasing body of evidence implicates angiogenesis in hematologic malignancies such as multiple myeloma, lymphoma, and CLL.[40,41] Angiogenesis factors such as basic fibroblast growth factor (bFGF) upregulate bcl-2, delaying programmed cell death. New antiangiogenesis agents available for clinical trials include thalidomide, SU5416, and SU6668.[42,43] SU5416 specifically inhibits VEGF signaling through the Flk-1 receptor in endothelial cells. SU6668 inhibits the VEGF receptor Flk-1/KDR; the platelet derived growth factor (PDGF) receptor-ß (PDGFR), and the fibroblast growth factor (FGF) receptor-1 tyrosine kinase. Both intravenous and

oral formulations of SU6668 are entering phase I trials.

The proteasome is a large, multicentric protease complex with a pivotal role in cellular protein regulation. The proteasome degrades proteins that have been conjugated to ubiquitin, resulting in what is referred to as the ubiquitin-proteasome pathway. The ubiquitin-proteasome pathway plays a critical role in the degradation of intracellular proteins involved in cell cycle control and tumor growth. Many tumor cells depend on rapid cell cycling which requires expression and degradation of numerous regulatory proteins. Cells accumulate in the G_2-M phase of the cell cycle with a decrease of cells in G_1. The proteasome is also required for activation of NFκB which plays a role in maintaining cell viability through the transcription of inhibitors of apoptosis. Since NFκB can induce drug resistance, this agent may make cells more chemosensitive.

PS-341, a dipeptidyl boronic acid, is a specific and selective inhibitor of the 26S proteasome.[44,45] It helps to eliminate damaged or misfolded proteins and plays a regulatory role in multiple cellular pathways involving cell cycle, transcription factor activation, and cell trafficking. PS-341 may also induce apoptosis. PS-341 has shown activity in cell types characterized by overexpression of BCL-2.

Increasing evidence supports the use of proteasome inhibitors in the therapy of patients with CLL. First, the ubiquitin-proteasome-dependent protein processing may be altered in CLL cells. In addition, proteasome inhibition has been shown to induce apoptosis of CLL lymphocytes at concentrations that do not have that effect on normal cells.[46-48] Activity has also been observed in multiple myeloma.[49]

A variety of promising new chemotherapy agents with unique mechanisms of action are currently being studied in clinical trials. Future strategies must be directed at attacking appropriate therapeutic targets using rational combinations of these drugs and other new compounds. It is important to accrue patients rapidly to clinical trials so that effective new approaches are quickly made available with the goal of curing patients with hematologic malignancies.

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Chronic Lymphocytic Leukemia
Chairmen: C. Bernasconi, F. Lauria

Chronic lymphocytic leukemia: therapeutic update

GUILLAUME DIGHIERO
Institut Pasteur, Paris, France

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Correspondence: Guillaume Dighiero, Institut Pasteur, F-75724 Paris, France

Chronic lymphocytic leukemia (CLL), the most frequent adult leukemia in the Western world, is a neoplastic disease of advancing age, characterized by a progressive accumulation of functionally incompetent, long-lived small mature monoclonal B lymphocytes, with a characteristic phenotype ($CD5^+$,$CD23^+$, low surface immunoglogulins and CD79b).[1] It is usually first recognized by the primary care physician. It is far from uniform in presentation and clinical course.[2,3] About one-third of patients never require treatment and have a long survival; in another third an initial indolent phase is followed by progression of the disease; the remaining third of patients have aggressive disease at the outset and need immediate treatment.[4]

The development of the Rai[2] and Binet[3] staging systems has allowed the division of patients with chronic lymphocytic leukemia into three prognostic groups: good, intermediate and poor prognosis (Table 1). Binet's good prognosis group (stage A, 63% of CLL patients with a 10-year survival of 51%) includes twice as many patients as Rai's stage 0, since it includes all Rai 0, 2/3 of Rai I and 1/3 of Rai II patients. Rai stage 0 patients, which includes 31% of CLL patients, display a 10-year survival of 59%. Rai's intermediate prognosis group includes 59% of CLL patients compared to 30% in Binet's intermediate group.[4] These differences can affect the design of clinical trials.

The two staging systems have improved the identification of patients who need immediate treatment. Two long-term French trials[4] and a meta-analysis of most randomized trials[5] demonstrated that therapy with chlorambucil, an oral alkylating agent and the standard treatment of chronic lymphocytic leukemia, could be deferred for Binet stage A patients. This low-risk group, which constitutes almost two-thirds of patients with chronic lymphocytic leukemia, has a median age at diagnosis of 64 years and an expected survival of >10 years, which is close to the life expectancy of a normal population matched for sex and age.[4,5] Moreover, deferring therapy until forced by disease progression does not compromise survival.[4,5] However, as shown in Table 2, over 25 % of these indolent cases die of causes related to chronic lymphocytic leukemia, 40 % progress to advanced stages, and 50 % ultimately require treatment.[4]

Neither the Rai nor the Binet staging system can predict which patients among the good prognosis group will shift into progressive disease. Serum levels of β_2-microglobulin, lactate dehydrogenase, and soluble CD23 (a B-cell membrane protein) can help predict disease activity, but the presence in the leukemic B cells of cytogenetic abnormalities such as 11q deletions,[6] or somatic mutations in the immunoglobulin heavy chain genes[1,7,8] are better predictors of rapid progression and survival. These recent results suggest that there are two types of chronic lymphocytic leukemia: one arises from relatively less differentiated (immunologically naive) B-cells with unmutated heavy chain genes, and has a poor prognosis; the other evolves from more differentiated B-cells (memory B-cells) with somatically mutated heavy chain genes, and has a good prognosis.[7-9]

Treatment decisions

Once the diagnosis of CLL has been made the treating physician is faced with the decision not only of how to treat the patient, but also when to initiate therapy. Criteria for initiating treatment may be quite different in clinical practice and in trials. A subset of patients are considered as having smoldering CLL; they include those with Rai stage 0 or Binet A disease. The two staging systems have improved the identification of patients who need immediate treatment. Two long-term French trials and a meta-analysis of most randomized trials demonstrated that therapy of chronic lymphocytic leukemia, could be deferred for Binet stage A

Table 1. Rai and Binet staging systems.

	Staging system	*Definition criteria*	*% of patients*	*Median survival*	*CLL related death*	*Treatment decision*
Good Prognosis	Rai 0	Lymphocytosis alone; Hb ≥ 11 g/dL and platelets ≥100x10^9/L;	31%	10 y survival 59%	27%	Wait and Watch until progression
	Binet A	Lymphocytosis, Hb ≥l0 g/dL and platelets ≥100x10^9/L; < 3 lymphoid areas (1) enlarged*	63%	10 y survival 51%	31%	Wait and Watch until progression
Intermediate Prognosis	Rai I+Ii	Lymphocytosis, Hb ≥l1 g/dL and platelets ≥100x10^9/L; Lymph-adenopathy (Stage I) spleen and/or liver enlarged nodes may or may not be enlarged (Stage II)	61%	96 mo		Treatment if progressive disease
	Binet B	Lymphocytosis,; Hb ≥l0 g/dL and platelets ≥100x10^9/L; three or more areas enlarged	30%	81 mo	>80%	Treatment in most cases
Poor Prognosis	Rai III+Iv	Lymphocytosis, hemoglobin < 110g/L (Stage III), platelets < 100x10^9/L (Stage IV), organomegaly may or may not be present organomegaly may or may not be present	8%	63 mo	>80%	Treatment in most cases
	Binet C	Lymphocytosis, platelets < 100x10^9/L, anemia and organomegaly may or may not be present	7%	60 mo	>80%	Treatment in most cases

Table 2. Rai and Binet good prognosis patients after 11-year evolution.

	% of patients	*10-year survival*	*% of patients without evolution*	*% of CLL related deaths*	*% of patients evolving to B or C*	*% of patients receiving treatment*
STAGE 0	31%	59%	57%	27%	32%	43%
STAGE A	65%	51%	47%	31%	41%	53%

patients and that deferring therapy until forced by disease progression does not compromise survival. However, these studies were conducted with alkylator-based regimens. The recommendations may change if new drugs are demonstrated to have an advantage in CLL.

Since long-term studies have shown that about one third of Rai stage 0 and Binet stage A patients die of CLL-related causes, early identification of these patients and their inclusion in clinical trials, remains an unsolved issue. It is unclear whether young patients with Binet stage A or Rai stage 0 whose leukemic B-cells express unmutated V genes or deleterious chromosomal abnormalities such as 11q deletions or alterations in the p53 protein, would benefit from early treatment. This possibility should be tested in a prospective clinical trial. By contrast, there is consensus that most patients with Binet stage B or C, or with Rai stage III or IV, and patients with Rai stages I or II with progressive disease[10] whose life expectancy does not exceed 7 years should be considered for early treatment. Although, most patients with Rai stages I and II with progressive disease and Rai stages III and IV or Binet stages B and C require treatment at presentation, some patients can still be monitored without therapy until they exhibit evidence of progressive or symptomatic disease.

But what treatment? Chlorambucil is the best-tolerated and least expensive drug. Fludarabine, a purine analog, yields the best response rates for a single agent but causes more myelosuppression and reductions in CD4 lymphocytes than chlorambucil, and costs more than combination chemotherapy.[11] Alopecia, vomiting, diarrhea, and cardiac toxicity are problems with combination chemo-

therapy which, like fludarabine, requires intravenous administration. None of these treatments can cure chronic lymphocytic leukemia. Rai *et al.*[12] reported the results of a long-term multicenter, randomized trial involving 509 previously untreated patients with advanced chronic lymphocytic leukemia in which fludarabine was compared with chlorambucil. Although the dose of chlorambucil was somewhat lower than usual, this important trial found higher response rates and longer durations of remission and progression-free survival with fludarabine than with chlorambucil. Nevertheless, neither fludarabine nor chlorambucil prolonged survival. A randomized trial by the French Group enrolled 938 previously untreated patients with stage B or C chronic lymphocytic leukemia.[13] Fludarabine was compared with two combinations: cyclophosphamide, adriamycin, and prednisone (CAP), and a modified version of cyclophosphamide, adriamycin, vincristine, and prednisone (mini-CHOP). Rates of response and progression free-survival were roughly similar for mini-CHOP and fludarabine, and better than the rates for CAP. Similar results were obtained in a European trial that compared fludarabine with CAP[14] and in a trial comparing cladribine plus prednisone to chlorambucil plus prednisone.[15] In addition, a meta-analysis of 10 randomized trials involving 2,035 patients with advanced chronic lymphocytic leukemia compared chlorambucil with several combination chemotherapy regimens;[5] in none of these trials was improvement in response rates translated into improved survival. The lack of improvement in survival despite superior response rates has been observed with other chronic lymphoid malignancies, and could be due to subsequent treatment or the failure of the treatment to eliminate all malignant cells.

Evaluations of treatments called intensification procedures, which aim at complete molecular remission (no evidence of molecular markers of the malignant clone after treatment), are in progress. They include purine analogs with or without other drugs[16] followed by autologous bone-marrow transplantation[17] or monoclonal antibodies (e.g., Campath[18,19] or anti-CD20,[20] or both. Some patients have entered a sustained molecular remission with such intense treatments, but it is unknown whether the treatment cures the disease or just delays a relapse.[21] Conventional allogeneic bone marrow transplantation can probably be curative in some cases, but only 10% of patients with chronic lymphocytic leukemia are eligible for this treatment, which has a >40% mortality rate. Allogeneic bone marrow transplantation in which the patient's marrow is not ablated by high-dose chemotherapy is another option under evaluation.[17]

Randomized trials of these strategies are time-consuming and expensive, but essential. Meanwhile, what should physicians do for patients with chronic lymphocytic leukemia? Patients with stage A (or stage 0) need only observation. In our opinion, a young patient (<65 years?) for whom a cure is possible in principle should participate in a randomized trial of one of the aggressive new strategies. For an older patient, or one with considerable co-morbidity, the aim should be palliation. For this we prefer chlorambucil. For patients who do not fit either category, therapeutic decisions vary. Some physicians, especially in Europe, prefer to start with chlorambucil and switch to mini-CHOP or purine analogs if there is no response, whereas other physicians initiate treatment with fludarabine. Fludarabine is the best option for patients refractory to alkylating agents[22] and we recommend mini-CHOP for patients who are refractory to fludarabine.[13] There is evidence that these patients with a poor prognosis may benefit from intensification strategies if their general health permits. We believe that, depending on age and co-existing diseases, intensification procedures are justified for these patients.

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Chronic Lymphocytic Leukemia
Chairmen: C. Bernasconi, F. Lauria

Campath-1H (alemtuzumab). A major new drug in chronic lymphocytic leukemia

MICHAEL J. KEATING
M.D. Anderson Cancer Center, Houston, TX, USA

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Correspondence: Michael J. Keating, M.D. Anderson Cancer Center, Houston, TX, USA.

Campath-1H (alemtuzumab) is a chimeric monoclonal antibody that binds to CD52, a surface antigen expressed in high density on the surface of B- and T-lymphocytes and on the majority of malignant cells from lymphomas. It is also expressed on monocytes, macrophages, eosinophils, and some epithelial cells of the male reproductive system. CD52 is a peptide of 12 amino acids attached to the cell membrane by a glycosylphosphatidyl-inositol (GPI) anchor and carrying a large oligosaccharide. Approximately $5x10^5$ molecules are present on the surface of lymphocytes.[1] Functional studies of antibody isotypes have identified human IgG1 as the most potent isotype for inducing complement activation and cell-mediated killing.[2] Campath-1H was developed using the variable regions of Campath-1G, a rat monoclonal antibody against CD52, grafted onto the constant region of human IgG1.

Mechanism of action

Campath-1H is lytic to cells that express CD52. Both antibody-dependent cellular cytotoxicity (ADCC) and the complement cascade appear to be important. Present evidence is leaning away from complement activation as the main mechanism of cell killing and moving towards ADCC as the *in vivo* mechanism of action. Campath-1H binds the full range of human IgG Fc receptors suggesting that target cell killing may be mediated by binding of Campath-1H to Fc receptors. There is some evidence at present of direct induction of apoptosis and this is another potential mechanism of action. The novel mechanism of Campath-1H suggests that it will be active in patients who are resistant to therapy with traditional chemotherapeutic agents. Campath-1H was explored in the treatment of non-Hodgkin's lymphoma, autoimmune conditions, transplant conditioning, and was noted in early studies to be active against chronic lymphocytic leukemia; this led to further studies in this condition.

Campath-1H in chronic lymphocytic leukemia

Campath-1H was studied in a series of clinical trials in Europe and one in the United States. In previously treated patients, the complete response rate varied from 4-23% and the overall response rate from 42-69%.[3,4] In early studies it was noted that profound immunosuppression occurred, with a number of patients developing opportunistic infections including herpes zoster, invasive aspergillosis, cytomegalovirus (CMV), candidiasis, and pseudomonas. These studies also demonstrated rapid clearing of the lymphocytes from the blood of almost all patients; bone marrow disease was also very susceptible to the effects of Campath-1H.

The prognosis of patients who have failed to benefit from fludarabine after having been exposed to alkylating agents is very poor. In a series of 147 such patients at the M. D. Anderson Cancer Center (MDACC), the response rate to any subsequent therapy was only 23% with 14% of patients dying in the first three months of therapy; the median survival was only nine months. Fludarabine refractoriness was defined as failure to obtain a complete or partial response to a fludarabine-containing regimen or relapse within six months of obtaining a response.[5] This led to the development of a pivotal clinical trial for fludarabine-refractory patients. This was a multicenter study with 93 fludarabine-refractory CLL patients entered from a number of institutions in the United States and Europe.[6] Campath-1H was given at a dose of 3 mg, 10 mg, and 30 mg on successive days according to tolerance and once a dose of 30 mg was reached, this was given three times a week for four to twelve weeks. All patients were premedicated with acetaminophen and diphenhy-

dramine. All patients were also given prophylactic therapy with trimethoprim-sulphamethoxazole (TMP-SMX) and famciclovir during therapy and for two months following Campath-1H treatment.

The overall response rate was 33%. Two patients (2%) obtained a complete remission (CR) and 29 a partial remission (PR). Fifty-five patients had stable disease. Among those patients with stable disease, antitumor response was seen in the blood in 97%, in the lymph nodes in 62%, in the liver in 73%, and in the spleen in 71%. There was a strong correlation between probability of response and lymph node size. The larger the lymph node, the less likely patients were to respond. The most common adverse events included rigors, nausea and vomiting, rash, fatigue, and dyspnea. The majority of these events were grade 1-2 in severity and occurred in the first week to 10 days of therapy.

Infections occurred in 76% of patients. One-third of the infections were grade 3-5 and included episodes of bacterial pneumonia, fungal infections, cytomegalovirus, septicemia, and Candida. Most infections occurred within the first two months of therapy. This trial demonstrated that Campath-1H was very effective in clearing disease from blood, bone marrow, and the spleen. Lymph node sites were relatively refractory. The overall median survival in this patient population was 16 months.

Minimal residual disease

Four-color flow cytometry (MRD flow) was developed as a highly sensitive technique to detect residual disease and can identify one tumor cell in more than 10,000 cells. Investigators in the UK treated 29 refractory CLL patients. Some had extensive disease and others had only minimal residual disease. Complete remission was noted in 34% of these patients and the overall response rate was 59%. Ten patients who achieved complete remission were evaluated with 4-color flow cytometry to identify whether they were MRD flow positive or negative. Five of the 10 patients achieving CR became MRD flow negative.[6]

There is some experience with Campath-1H in previously untreated patients with CLL and these studies are continuing. Osterborg and colleagues studied this agent in nine previously untreated patients with CLL. Campath-1H was administered either subcutaneously or intravenously three times a week for a maximum of 18 weeks. An initial dose of 3 mg per injection was escalated rapidly to 10 mg, then 30 mg. Three patients obtained a complete remission and another five patients a partial remission. Hematologic toxicity was mild. Prolonged lymphopenia occurred but serious infections were noted in only one patient.

Campath-1H for T-cell prolymphocytic leukemia

Campath-1H is being studied for use in T-cell prolymphocytic leukemia (T-PLL) and has shown favorable results. Twenty-two relapsed or refractory T-PLL patients were treated with Campath-1H. The overall response rate was 77% with 59% of patients obtaining a CR. The median disease-free interval was nine months. Survival was significantly prolonged in patients who achieved a CR (median 13.5 months) compared to a PR (median 6 months).[8] In an ongoing study at MDACC 2 of 4 patients with T-PLL achieved a CR.[9]

Toxicity

Campath-1H is minimally associated with infusion-related toxicity. Fever and rigors are most common but most of these reactions are grade 1-2. The incidence of grade 3-4 fever and rigors is approximately 10-15%. Another 10-15% of patients develop rashes which are typically urticarial. Nausea and vomiting can occur but are usually mild. Campath-1H toxicity increases as the dose is escalated to 30 mg but after the first 2-3 doses of 30 mg the side effects are markedly decreased. Anecdotal reports suggest that premedication with corticosteroids is effective in modifying the side effects but this regimen should not be continued for long periods of time.

CD4 and CD8 counts are profoundly depressed and often reach 0 after the first 1-2 weeks of therapy. As treatment continues, T-cells begin to repopulate the peripheral blood. With the T-cell immunosuppression, patients in earlier studies were noted to have a high frequency of herpes simplex and herpes zoster reactivation and some developed *Pneumocystis carinii* pneumonia and cytomegalovirus infection. In more recent studies, all patients receive prophylaxis with TMP-SMX and antiviral medications such as acyclovir, famciclovir, or valacyclovir. Despite the fact that CD52 is not present on hematopoietic stem cells, anemia, thrombocytopenia, and neutropenia occur in 25-40% of patients. In one study neutropenia occurred in 48% of patients and thrombocytopenia in up to 39% of patients. This is more common in patients with advanced Rai stage on presentation.[10]

More extensive experience is being obtained with Campath-1H in both B- and T-cell leukemias. In two recent publications, a high response rate was noted in patients with advanced and refractory T-cell PLL.[11] In addition, studies continue using Cam-

path-1H to treat minimal residual disease. In an ongoing trial at MDACC, 24 patients with minimal disease after chemotherapy received Campath-1H 10 mg three times a week for one month. A subsequent cohort of 10 patients received 30 mg three times a week. The features noted in this preliminary analysis are that reduction in lymphocytes occurs in almost every patient. Peripheral blood is cleared if circulating malignant cells are present at the start of therapy. Bone marrow nodules are eradicated in 75% of patients. Lymph node enlargement is more resistant to therapy with few patients obtaining a complete remission. Bone marrow becomes flow negative in 80% of patients and 50% of patients who are polymerase chain reaction (PCR) positive in the bone marrow at initiation of therapy became PCR negative. Reactivation of cytomegalovirus occurred even at the lower dose of 10 mg.

As Campath-1H is most effective in treating disease in bone marrow and MabThera (rituximab) is effective in lymph node disease and less effective in blood and bone marrow, combination studies of these agents appear logical and are already in progress. There appears to be no unusual toxicity with the combination.

Prospectives

Administration of Campath-1H is being investigated not only intravenously but also by the subcutaneous route. There is evidence that soluble CD52 is present in plasma and this may neutralize some of its efficacy. Soluble CD52 is associated with adverse parameters including advanced stage, bulky disease, and extensive prior therapy. There is little pharmacologic information about Campath-1H treatment and as this information becomes available, more rational doses and schedules may be tailored. Studies in minimal residual disease will obviously continue as will studies in previously untreated patients.

The subcutaneous route of administration appears to be well tolerated and in preliminary studies has a response rate of approximately 80% in previously untreated patients. The subcutaneous route of administration is associated with mild to marked local reactions. There is little information available at present regarding the blood levels of Campath-1H achieved with the subcutaneous route of administration.

Conclusions

Campath-1H is the most lympholytic agent noted in clinical trials in CLL. The high response rate and improved survival in advanced stage patients suggests that Campath-1H may have an application in patients and will be used more frequently as earlier therapy. The comparison of the subcutaneous and intravenous routes for safety and toxicity will be of interest. Combining Campath-1H with other monoclonal antibodies and chemotherapeutic agents is rational and should provide interesting data in the future. Campath-1H has emerged as the most active agent explored in T-cell prolymphocytic leukemia.

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Chronic Lymphocytic Leukemia

Chairmen: C. Bernasconi, F. Lauria

Nelarabine: a new drug for hematologic malignancies

CARLOS O. RODRIGUEZ, JR.* VARSHA GANDHI*°
*Department of Experimental Therapeutics and °Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Correspondence: Varsha Gandhi, Ph.D., Department of Experimental Therapeutics, Box 71, 1515 Holcombe Blvd. Houston, TX 77030, USA. Phone: international +1.713.7922989. Fax: international +1.713.7944316. E-mail: vgandhi@mdanderson.org

The discovery of purine nucleoside phosphorylase (PNP) deficiency, the elucidation of its pathophysiology, and the observation of T-specific lymphopenia in this disease provided the rationale for the development of analogs of deoxyguanosine (dGuo). Metabolic studies demonstrated that through the action of specific kinases, nucleosides are trapped intracellularly in the form of 5'-phosphates selectively in thymus-derived lymphoblasts or peripheral lymphocytes obtained from humans.[1] In addition to the high levels of specific kinases, other studies demonstrated low levels of 5'-nucleotidases, which could degrade the 5'-phosphates and thus maintain high concentrations of dGuo triphosphate (dGTP) in T-lymphoblasts.[2] Although favorable levels of kinases and nucleotidases were present in T-lymphoid cells at all stages of development, the immature T-cells showed the most favorable ratio of these enzymes, making them most susceptible to dGuo-mediated toxicity.[3] Consistent with these results, cultured T-lymphoblasts accumulate higher levels of intracellular dGTP from dGuo than do normal T-lymphocytes and leukemic T-cells.[4] Additional mechanistic studies using dGuo demonstrated that the elevated levels of dGTP resulted in a dGTP-mediated inhibition of DNA synthesis and cell death.[5,6] This information provided the impetus for biochemical, metabolic, and *in vitro* pre-clinical studies.

These observations suggested that inhibitors of PNP may serve as T-cell selective agents. In addition, dGuo analogs that are resistant to PNP degradation may be effective for T-cell specific diseases. Because during this period, analogs of deoxycytidine (dCyd) such as arabinosylcytosine (ara-C) and deoxyadenosine (dAdo) such as arabinosyladenine (ara-A) were showing promise in the clinic, an obvious dGuo analog was arabinosylguanine (ara-G).

In contrast to both dCyd and dAdo analogs, which have demonstrated clinical utility, dGuo analogs were not developed until the synthesis of ara-G. This analog was synthesized by Reist and Goodman[7] when it was shown that ara-C[8] and ara-A[9] were highly toxic in cell culture. Ara-G differs from its congener, dGuo, in that the carbohydrate moiety is an arabinose sugar (Figure 1). Brink and LePage reported the first studies of the biological activity of ara-G.[10]

Because dGuo was highly associated with T-cell specific toxicity, comparative experiments of ara-G-mediated sensitivity in whole cell systems have been accomplished. In contrast to other nucleoside analogs but similar to dGuo, Carson demonstrated that ara-G was toxic to normal immature T-cells with relative selectivity.[11] In cell lines,[12-14] ara-G demonstrated selective cytotoxicity to malignant T-lymphoid cells compared to mature T-cells,[15] non-T, non-B (null) cells or normal B-lymphoblasts,[12] malignant B-lymphoid cells,[13-16] or promyelocytes.[15] Consistent with the cell line data, the toxicity of ara-G in freshly isolated T-lymphoid, B-lymphoid, and myeloid leukemia cells showed T-lymphoblast selectivity.[15] Accompanying cellular metabolic investigations demonstrated that the accumulation of ara-G 5'-triphosphate (ara-GTP) was greatest in cells of T-lineage. The differential accumulation and consequent selective cytotoxicity was postulated to be due to the higher accumulation and retention and slower elimination of ara-GTP.[16]

Studies have demonstrated that ara-G permeates T-lymphoblastoid cells via nitrobenzylthioinosine-sensitive and –insensitive equilibrative nucleoside transporters.[17] Once inside the cell the rate-limiting step in the accumulation of the cytotoxic metabolite, ara-GTP, is dependent on the affinity of the enzymes responsible for the synthesis of the triphosphate (Figure 2). Generally the

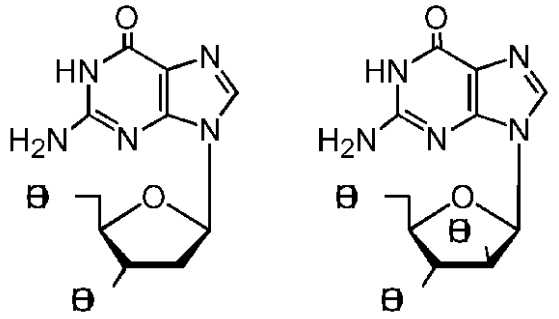

Figure 1. Structures of deoxyguanosine and arabinosylguanine. Note that the only difference between the native dGuo and ara-G is in the carbohydrate moiety.

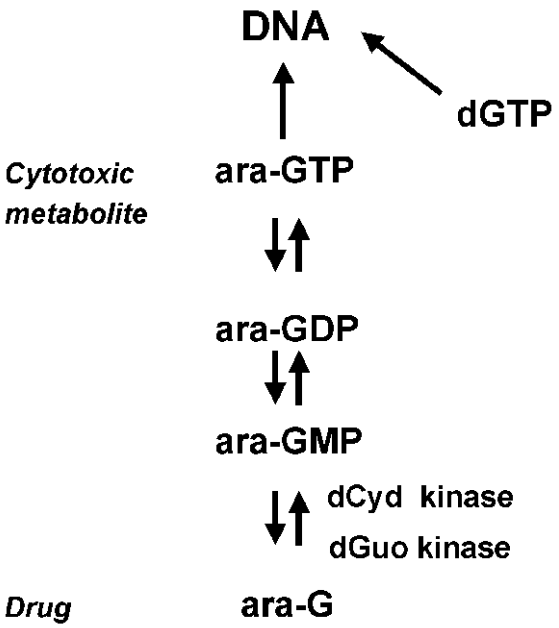

Figure 2. Metabolic activation schema of ara-G. The drug, ara-G is phosphorylated in the initial and rate-limiting step in the accumulation of the ara-GTP by cytoplasmic dCyd kinase and mitochondrial dGuo kinase. The cytotoxic metabolite, ara-GTP competes with dGTP for incorporation into DNA. Incorporation into DNA is the critical event that mediates ara-GTP-induced apoptosis.

rate-limiting step in the generation of the triphosphate is the phosphorylation of the nucleoside analog to its respective monophosphate. Using purified protein or crude cell extracts in cell-free systems it has been shown that for ara-G, this critical first step is catalyzed by both dCyd kinase[16,18,19] and dGuo kinase.[20] Although these *in vitro* investigations demonstrated that both dCyd kinase and dGuo kinase are able to phosphorylate ara-G, controversy exists with respect to the role of each of these enzymes in whole cells. Using a dCyd kinase-deficient mutant of the T-lymphoblastic cell line CEM,[21] we showed that there was accumulation of intracellular ara-GTP demonstrating that the presence of only dGuo kinase could result in the accumulation of intracellular ara-GTP. When this cell line was transfected with dCyd kinase, or constructed to overexpress dGuo kinase, there was a concomitant increase in the accumulation of intracellular ara-GTP both in a concentration- and time-dependent manner. Interestingly, at low concentrations of ara-G, the activity of dGuo kinase appeared to predominate whereas at higher concentrations of ara-G, the activity of dCyd kinase appeared to play a more prominent role in the accumulation of intracellular ara-GTP.[21]

After ara-G is metabolized to ara-G monophosphate, it is further phosphorylated to di- and then triphosphate by nucleoside monophosphate and diphosphate kinases, respectively. Once ara-GTP is formed, it competes with dGTP for incorporation into DNA. Previous work has shown that only DNA synthesis is inhibited by ara-G without there being any effect on RNA synthesis.[22,23] In a primer extension assay system designed to model *in vitro* DNA synthesis by using purified DNA polymerase α, the K_m values for incorporation into DNA for dGTP and ara-GTP were 0.02 and 0.2 μM, respectively.[24] Although there is a 10-fold difference in K_m values, the favorable concentration of cellular ara-GTP compared to that of dGTP suggests a high possibility of DNA incorporation of the analog. Additional *in vitro* work has shown that after incorporation, DNA primers containing ara-GTP are resistant to both further elongations in either replication or repair reactions by purified enzymes *in vitro*.[24] These findings suggest that ara-GTP competes with its native nucleotide, dGuo triphosphate, for incorporation into DNA.[23,25,26] Recent investigations in whole cells showed that blocking ara-GTP incorporation into S-phase DNA abolished biochemical and morphologic features of apoptosis, even in the presence of cytotoxic levels of ara-GTP. These data were consistent with the *in vitro* data and demonstrated that

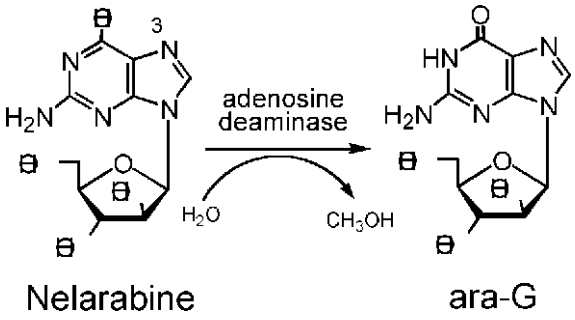

Figure 3. Structures of nelarabine and ara-G. Nelarabine, the prodrug, is a 6-methoxy derivative of ara-G that is demethoxylated by adenosine deaminase to form ara-G.

incorporation of ara-GTP into DNA is the critical event that mediates the induction of apoptosis in CEM cells.

Despite these compelling data for the use of ara-G in the treatment of T-acute lymphoblastic leukemia, its low water solubility and difficulty in synthesis kept this analog out of the clinic. The clinical debut of ara-G has been hastened by the development of nelarabine (compound GW506U78, 2-amino-6-methoxy-arabinofuranosylpurine), which serves as a pro-drug for ara-G (Figure 3). Nelarabine is a 6-methoxy derivative of ara-G, which is more water-soluble than ara-G and is demethoxylated by adenosine deaminase (ADA) to produce ara-G.[23,27] The presence of high specific activity of ADA in plasma and large body organs suggested that the conversion of nelarabine to ara-G should be efficient in animals or humans.[23]

Based on the findings in the first clinical trial and the rationales presented above, the clinical efficacy of nelarabine was and is being evaluated in additional phase I and II clinical trials.[28-30] Nelarabine proved effective in the treatment of multiple relapsed or refractory hematologic diseases including myeloid, acute T-cell or B-cell lymphoblastic and indolent T-cell or B-cell lymphocytic diseases. The associated plasma pharmacokinetics of nelarabine and ara-G was evaluated in 71 (25 pediatric and 46 adult) patients on the first day of therapy.[31] The plasma pharmacokinetic data can be summarized as follows.

First, the harmonic mean half-life ($t_{1/2}$) of nelarabine in pediatric and adult patients was 14.1 minutes and 16.5 minutes, respectively, suggesting a rapid elimination and/or conversion of nelarabine to ara-G. Second and consistent with the first observation, the maximum concentrations (C_{max}) of ara-G occurred at or near the end of the nelarabine infusion indicating a fast conversion of nelarabine to ara-G. Third, the accumulation of ara-G was dose-dependent and the C_{max} of ara-G ranged from 11.6 µM to 308.7 µM at nelarabine doses ranging from 5 mg/kg to 75 mg/kg. Fourth, the dose-normalized C_{max}, time of the C_{max}, and the steady-state volume of distribution of ara-G were similar in children and adults. However, the clearance of ara-G was higher in pediatric patients (0.312 $Lh^{-1}kg^{-1}$) than in adult patients (0. 213 $Lh^{-1}kg^{-1}$). This difference was significant ($p< 0.001$) and may be due to the elimination half-life of ara-G, which was shorter in pediatric patients than in adult patients (2.1 hours vs 3.0 hours; $p <0.01$). Fifth, there was no statistically significant difference in the pharmacokinetics of nelarabine between any of the groups of patients with diseases of different cell lineage suggesting that all leukemia and lymphoma patients have a similar capacity to convert nelarabine and retain ara-G.

While plasma pharmacokinetic data suggested no lineage specific differences in the concentration of nelarabine or ara-G in plasma, evaluation of the cellular pharmacology provided evidence for lineage selectivity. The cellular pharmacokinetic data[28] demonstrated that the median intracellular ara-GTP concentration of responders was significantly greater than that of the non-responders (ara-GTP = 120 µM and 50 µM, respectively; $p = < 0.0001$). Consistent with previous *in vitro* data, the responders in the phase I clinical trial tended to have T-lymphoblastic malignancies whereas non-responders tended to have B-lymphoblastic, T-lymphocytic or myeloid malignancies.[28,31,32] These clinical investigations suggest an immature T-cell (T-lymphoblasts) selective sensitivity to ara-G.

Because the first phase I trial demonstrated a strong linear relationship between intracellular ara-GTP and response to nelarabine therapy, a pilot protocol was designed to evaluate the efficacy of fludarabine with nelarabine in a test of the biochemical strategy in patients with hematologic malignancies. The cellular pharmacokinetics was investigated to seek a relationship between response and accumulation of ara-G triphosphate (ara-GTP) in circulating leukemia cells and to evaluate biochemical modulation of cellular ara-GTP metabolism by fludarabine triphosphate. Nine of the 13 total patients had indolent leukemias,

including six whose disease had failed to respond to prior fludarabine therapy. Two patients had T-acute lymphoblastic leukemia, one had chronic myelogenous leukemia, and one had mycosis fungoides. Nelarabine (1.2 g/m^2) was infused on days 1, 3, and 5. On days 3 and 5, fludarabine (30 mg/m^2) was administered 4 hours before the nelarabine infusion. Plasma and cellular pharmacokinetic measurements were conducted during the first 5 days. Seven patients had a partial or complete response, six of whom had indolent leukemias. The disease in four responders to this pilot protocol had failed to respond to prior fludarabine therapy. As observed in the first phase I study, the median peak intracellular concentrations of ara-GTP were significantly different (p =0.001) in responders (890 µM, n = 6) and non-responders (30 µM, n = 6). The cellular elimination of ara-GTP was slow (median, 35 hours; range, 18 to > 48 hours). The ratio of ara-GTP to its normal counterpart, deoxyguanosine triphosphate, was higher in each patient (median, 42; range, 14 to 1,092) than that of fludarabine triphosphate to its normal counterpart, deoxyadenosine triphosphate (median, 2.2; range, 0.2 to 27). In summary, fludarabine plus nelarabine was an effective, well-tolerated regimen against leukemias. Clinical responses suggested the need for further exploration of nelarabine against fludarabine-refractory diseases. Additionally, the determination of ara-GTP levels in the target tumor population may provide a prognostic test for the activity of nelarabine.

In conclusion, the phase I trials evaluating the efficacy of nelarabine alone and in combination with other nucleosides such as fludarabine provide the rationale and initial responses necessary to activate interest in this drug and seek FDA approval for its use as frontline therapy. Because T-cells appear to be particularly sensitive to the cytotoxic effects of nelarabine, this analog should be evaluated in other T-cell diseases such as T-prolymphocytic leukemia, mycosis fungoides and Sézary 's syndrome, and acute and chronic graft-versus-host disease. Finally combinations of nelarabine with other chemotherapeutic agents or treatment modalities should be explored.

Acknowledgments

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Chronic Lymphocytic Leukemia

Chairmen: C. Bernasconi, F. Lauria

Chronic lymphocytic leukemia: treatment goals and current therapy

EMILI MONTSERRAT
Institute of Hematology and Oncology, Department of Hematology Hospital Clínic, University of Barcelona, Barcelona, Spain

Correspondence: Emili Montserrat, M.D., Department of Hematology Hospital Clínic, University of Barcelona, Villarroel, 170 08036, Barcelona, Spain. Phone/fax: international + 34.9.32275475. E-mail: emontse@clinic.ub.es

Chronic lymphocytic leukemia (CLL) is a disease with a variable clinical course and one for which there is not a totally effective, curative treatment. Because of this, risk-adapted treatment strategies are required, the goals of therapy being to improve the quality of life and to prolong survival. Due to the variable impact of the disease on survival, treatment decisions cannot be made without taking into consideration the patient's prognosis.[1]

For many decades the treatment of CLL has revolved around the use of alkylating agents, particularly chlorambucil. This resulted in palliation of symptoms but no improvement in survival. A number of trials conducted in the last two decades allowed some conclusions to be established regarding CLL therapy: i) for patients with low-risk disease (Binet A, Rai 0), there is no advantage of early, immediate treatment with chlorambucil as compared to a *wait and see* approach followed by treatment upon disease progression;[2,3] ii) in patients with advanced disease (Binet B, C; Rai I to IV), combination chemotherapy produces higher response rate (RR) and longer freedom from progression (FFP) than chlorambucil but not longer survival.[3]

Recently, new and more effective treatments for CLL have been introduced. These include purine analogs (i.e. fludarabine, cladribine, pentostatin), monoclonal antibodies (i.e., Campath 1H, rituximab), hematopoietic stem cell transplants (HSCT), and immune manipulation. Purine analogs, particularly fludarabine, are the most effective agents in CLL. A number of trials have shown a higher RR and a longer FFP with fludarabine or cladribine than with chlorambucil or regimens such as COP, CHOP or CAP but, unfortunately, no significant differences in survival.[4-7]

Monoclonal antibodies, particularly Campath-1H, induce a significant number of responses, including complete response (CR), even in patients refractory to purine analogs.[8-10] Interestingly, Campath 1H has a selective effect on peripheral blood; this makes this agent useful as an *in vivo* purging tool for autologous stem cell transplants.[10] Rituximab has limited effect when given alone at standard doses but shows a dose/response effect and acts synergistically with fludarabine.[11,12] The efficacy of purine analogs given in combination with other cytotoxic agents (e.g. cyclophosphamide, mitoxantrone) and/or monoclonal antibodies is being extensively investigated. Preliminary results indicate that these approaches are more effective than purine analogs alone, with impressive results having been reported with fludarabine combined with other cytotoxic agents and/or monoclonal antibodies.[13-15]

An increasing number of subjects with CLL are being offered HSCT.[16,17] Autologous transplants do not cure the disease but may prolong survival in selected patients. In contrast, allogeneic transplants can cure about 40% of the patients, but at the cost of a high toxicity and mortality (transplant-related mortality (TRM): 25-50%). Because of this and the advanced age of most patients with CLL, the role of allotransplants with reduced intensity conditioning regimens is being intensively investigated; preliminary results are encouraging, with TRM of 10-20% (at 1 year) and overall survival of about 60% (at 1 year). Finally, it is now possible to manipulate the immune system of patients with CLL to restore T-cell immunosurveillance mechanisms over the neoplastic B-cell population. As a consequence of all these advances,

Table 1. CLL: response rate and overall survival over the years.

Year	*Treatment*	*CR (%)*	*Molecular responses*	*Median S. (yrs.)*
1960	Chlorambucil	< 5	no	5
1970	COP	10-20	no	5
1980	CAP, CHOP	15-25	no	5
1990	Purine analogs	25-40	sporadic	6-8
2000	PA + others	40-60	frequent	8-10
	Stem-cell transplants	70-80	very frequent	

the rate of CR, including molecular CR, in CLL patients has been dramatically increasing during the last decade. In addition, the survival of patients with CLL has also been improving (Table 1). It is to be hoped that a significant proportion of patients with this form of leukemia will become curable in the next decade.

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Aggressive Lymphomas

Chairmen: M. Baccarani, G. Torelli

Aggressive lymphomas: therapeutic update

Massimo Magagnoli, Monica Balzarotti, Armando Santoro
Department of Medical Oncology & Hematology, Istituto Clinico Humanitas, Milan, Italy

Correspondence: Armando Santoro, MD, Department of Medical Oncology & Hematology, Istituto Clinico Humanitas, Milan, Italy

The incidence of non-Hodgkin's lymphoma (NHL) has been rising at a rate of 4% per year in the last decades in the Western world. The cause of this increased incidence is not clear, and research in lymphoma epidemiology is currently ongoing. About half of all patients have histologically aggressive NHL,[1] and despite the fact that a large number of lymphomas share an aggressive clinical behavior, the term aggressive NHL usually refers to the *diffuse large B-cell lymphoma* (B-DLCL) of the REAL classification.[2] In 70-75% of cases aggressive NHL presents in an advanced stage (i.e. Ann Arbor stage stage III or IV) and is fatal if untreated. Although many patients with B-DLCL enjoy long term disease-free survival with anthracycline-based therapy, over half of them either do not obtain a complete remission (CR) or relapse after remission. Actually, the prognosis of a patient can be predicted at the time of initial diagnosis by the International Prognostic Index (IPI). The IPI score[3] identifies age, tumor stage, performance status, serum lactate dehydrogenase, and number of extranodal sites involved at diagnosis as independent risk factors. By combining the number of these factors at diagnosis, four risk groups can be identified with different probabilities of five-year survival when treated with standard-dose, anthracycline-based chemotherapy (0-1 factors, low-risk: 73%; 2 factors, low-intermediate-risk: 51%; 3 factors: high-intermediate-risk: 43%; 4-5 factors, high-risk: 26%). It should now be standard practice to use the IPI score for selection of appropriate therapy and when designing or evaluating the results of clinical trials.

Early stages

At present, the gold standard treatment for localized stage I and II disease is a short term combination of chemotherapy (CHT) and radiotherapy (RT). This definition comes from several controlled trials[4,5] and has been confirmed by two large randomized studies addressing the issue of whether RT can be avoided. The first study[6] compared eight courses of CHOP to three courses of CHOP plus involved field (IF) RT at doses of 40 to 55 Grays. Two hundred patients were treated in each arm: despite complete remission (CR) rates being similar in the two groups (73% vs 75%), patients treated with combined therapy showed a better five-year freedom from progression (FFP) (77% vs 66%) and overall survival (OS) (82% vs 72%). The second trial,[7] comparing eight courses of CHOP with or without RT, also showed a better FFP (73% vs 58%) and OS (84% vs 70%) for patients treated with the combined modality approach. As over 80% of patients with early stage disease can actually be cured, further effort should focus on reducing the acute toxicity and the treatment period rather than on improving the outcome.

Advanced stages

It is now 30 years since the development of the CHOP regimen and apparently no further progress has been made in terms of standard treatment for aggressive advanced stage NHL, despite many attempts having been made throughout this long period.[8] The first effort to improve treatment results over CHOP in the past years consisted in the inclusion of new active drugs in the standard regimen, as suggested by Goldie and Coldman's hypothesis.[9] However, the appealing data achieved in pilot studies with either second or third[10] generation regimens were not confirmed by series of randomized trials.[11,12] In particular the SWOG trial[13] comparing CHOP versus either MACOP-B, ProMACE-CytaBOM or m-BACOD definitively closed the debate strongly supporting CHOP as the gold-standard for high-grade NHL. However, as fewer than 50% of all patients with advanced disease can be cured (and less than 30% of those with

high risk IPI), it is essential to develop new therapeutic strategies for patients with advanced-stage and high-risk NHL. In recent years, further approaches have been investigated: a) intensification of conventional treatment; and b) front-line high dose chemotherapy (HDT) with peripheral blood stem-cell (PBSC) support.

a) Intensification of conventional treatment. Several phase I/II trials testing the intensification of CHOP or CHOP-like regimens have been published. These studies show the feasibility of increasing the dosage of doxorubicin and cyclophosphamide by up to two-fold[14,15] the standard CHOP. The ACVBP combination (doxorubicin, cyclophosphamide, vindesine, bleomycin and prednisone given every two weeks) of the GELA group, utilized in more than 3,000 patients, represents the first example of intensified CHOP-like regimen with an average dose-intensity of 2.2 as compared to standard CHOP.[16] The Boston Group evaluated a *high-dose* CHOP with the maximum-tolerated doses being cyclophosphamide 4 g/m^2 and doxorubicin 70 mg/m^2. Nineteen of 22 patients achieved CR, and 69% of all patients were progression-free at a median follow-up of 20 months.[17] Our group published the results of a dose-finding trial concerning a step-by-step dose intensification of doxorubicin and cyclophosphamide of the CHOP regimen.[18] Maximum tolerated dose was identified at 2,750 mg/m^2 for cyclophosphamide and 75 mg/m^2 for doxorubicin, with the courses given to outpatients every three weeks with granulocyte colony-stimulating factor (G-CSF) support. Considering the series as a whole, the 3-year CR rate, FFP and OS for the 42 patients with advanced disease were 86%, 98% and 60%, respectively. In a further study from our group and that of Aviano (manuscript in preparation), the intensified CHOP regimen was given every two weeks with cyclophosphamide 1,750 mg/m^2, doxorubicin 75 mg/m^2 and G-CSF support. This schedule was chosen as these were the maximum deliverable doses without growth factor in the first trial. Even in this case, we attempted to increase the cyclophosphamide dose in a step-by-step fashion by 250 mg/m^2 per dose-level. Nevertheless, the starting dose emerged as the maximum tolerated dose deliverable on a large scale. Thus, a total of 53 patients were treated with 1,750 mg/m^2 of cyclophosphamide and 75 mg/m^2 of doxorubicin every two weeks. Treatment was globally well tolerated with only 11% of administered cycles requiring day-hospital or ward admission for toxicity, frequently concerning transfusional support. Overall, 74% of patients achieved CR, and among cases with intermediate-high risk IPI score 12-month FFP and OS were 58% and 71%, respectively. These results compare well with those of the above-mentioned trials, with the advantage of an outpatient-only treatment administration. All these studies clearly show that it is possible to approximately double the dose-intensity of the old standard CHOP. Whether or not this approach will ameliorate the results of CHOP is too early to assess.[19] So far, the few prospective randomized comparisons do not suggest a positive rate for the increased dose-intensity. Nevertheless these trials concern low-risk cases[20] or do not plan a real increase of dose-intensity from the very beginning of the treatment program, as is the case of the escalated BACOP prompted by Meyer *et al*.[21]

b. Front-line HDT with PBSCT. Based on its efficacy in relapsing patients, HDT has also been tested in patients with *de novo* NHL . Gianni *et al.* conducted an *early intensification* trial on 75 patients with poor-risk aggressive NHL who were randomized to receive MACOP-B or high-dose chemotherapy (HDT) regimen with PBSC support.[22] After a median follow-up of 43 months, there was a statistically significant improvement in relapse-free survival (RFS, 93% vs 68%) and FFP (88% vs 42%) in favor of the HDT arm. Two further trials concerned *late intensification* after conventional chemotherapy and retrospectively analyzed the prognostic impact of the IPI score. In the GELA trial, complete responders to initial standard induction therapy were randomized to receive consolidation high-dose therapy and autologous bone marrow transplantation or additional conventional dose therapy.[23] After a median follow-up of 53 months, low-risk and low-intermediate risk patients had similar outcomes when treated with either of the two regimens, while high-intermediate and high risk cases showed better outcome when treated with the high-dose regimen (RFS 57% vs 36%, OS 65% vs 52%). The *Italian Non-Hodgkin's Study Group* randomized 124 patients with advanced disease to receive standard induction therapy alone (VACOP-B) or followed by autologous bone marrow transplantation.[24] Again, no difference in RFS or FFP could be detected for the whole series, whereas there was a statistically significant improvement in DFS (p=0.008) and a favorable trend in FFP (p=0.08) for high risk cases retrospectively evaluated according to IPI. Other-

wise, the preliminary results from two unpublished studies did not seem to show any benefit from HDT in this patient subset. The GELA reported preliminary results of the LNH93-3 study, which randomized high-intermediate and high-risk patients to a sequential chemotherapy program versus a short, intensive induction chemotherapy followed by PBSCT.[25] Patients undergoing full-course conventional induction therapy seemed to show better outcome than those receiving early HDT and therefore the study was closed before the planned accrual was completed. It must be outlined that in this study, the *high-dose* arm was likely and paradoxically less intensive than the *conventional dose* arm. A *German High Grade Lymphoma Study Group* randomized 312 patients to receive either 5 cycles of standard induction therapy or 3 cycles of standard induction therapy followed by HDT. In an initial analysis, the 2-year overall survival of patients in the two treatment arms was comparable.[26]

Of interest, considering all definitive and preliminary data, the two *positive* randomized trials included full-course standard induction therapy with or without CR requirement followed by HDT, whereas the two *negative* studies contained abbreviated standard induction therapy and early HDT. These data support the hypothesis that only high-intermediate and high-risk patients with aggressive lymphoma with at least a very good PR after standard induction therapy could benefit from the addition of high-dose therapy. Currently, in the United States an intergroup trial is testing this hypothesis. Patients with high/intermediate or high risk disease receive four cycles of CHOP therapy and, if at least PR is documented, are then randomized to receive either four additional cycles of CHOP or one additional cycle of CHOP followed by HDT with PBSC support. If the benefit of HDT is confirmed, subsequent trials will aim to improve response rate to induction chemotherapy in order to increase the number of patients suitable for high-dose therapy.

Relapsed/resistant disease

The randomized PARMA study stated that HDT is standard approach in relapsed eligible patients.[27] In this trial, patients relapsing after initial CR received two courses of conventional salvage chemotherapy (DHAP) and were then randomized to receive HDT or further DHAP chemotherapy. The FFP and OS rate were 46% and 53% among patients receiving HDT as compared with 12% and 32% for those receiving standard-dose chemotherapy, respectively (p=0.001). The results were tightly correlated to IPI score: no difference could be detected between the two arms for patients with no adverse factors, whereas intensive therapy was better for all other IPI scores > 1.[28] Apart from the clear therapeutic advantage of HDT, this study suggests that only responding patients can benefit from HDT. Thus, improvement in response rate with new induction regimens is warranted.

The management of primary resistant high-grade NHL remains of major concern. Indeed, only a small fraction of patients with this type of disease obtain an objective response to salvage treatment and even after HDT 3-year FFP and OS range from 0% to a maximum of 25% for cases with chemosensitive disease at progression.[29-31]

These data strongly support the need for newer approaches, such as new combinations of drugs, association of monoclonal antibodies and chemotherapy or allogeneic bone marrow transplantation.

Based on the encouraging results in indolent lymphoma, the anti-CD20 monoclonal antibody rituximab (Rituxan or Mabthera) has been tested in relapsed or refractory high-grade lymphoma producing a response rate of 30%. In a phase-II trial, the association of CHOP and rituximab was tested in 33 previously untreated patients, 29 of whom are alive and disease-free with a minimum of 2 years of follow-up.[32]

The so-called *mini* allogeneic transplantation consists in an immunologic approach in which non-myeloablative chemotherapy and/or radiation is given as a conditioning regimen with the aim of reducing extra-hematologic toxicity, morbidity and mortality, of inducing sufficient immunosuppression to avoid graft rejection, and of reducing the *cytokine storm* and thus graft-versus-host disease (GvHD). The efficacy of this approach has not yet been determined in aggressive lymphomas, but recent reports are promising. Kouri *et al.* treated 15 patients with relapsed/refractory aggressive lymphomas. There was a 53% complete remission rate and a 13% partial response rate in this group with a median follow-up of six months.[33] As the transplant-related mortality rate seems far less than in classical allogeneic bone marrow transplantation, further exploration of this strategy is highly recommended.

Non-Hodgkin's lymphoma in the elderly

Approximately 45% of patients with NHL are older than 60 years at diagnosis. The difficulty in treating these patients arises from a number of factors: age, hematologic tolerance, need to reduce dose.

First of all, age over 60 has been recognized as a poor prognostic factor in the IPI. Moreover, elderly patients show poor hematologic tolerance, even though a direct correlation with chronological age is not so clear, and thus the dosages of key drugs (cyclophosphamide and anthracyclines) are often reduced in these patients, with a decrease of efficacy.

In an analysis by Gomez *et al.*[34] concerning 267 patients with a median age of 70 years treated with CHOP, mortality during chemotherapy was 19%, and 82% of deaths were due to infections (independently of neutropenia). In multivariate analysis, ECOG performance status (2 to 4) emerged as the only predictive factor for mortality, whereas chronological age and doxorubicin dosage were not correlated with fatal toxicity. Several studies have been specifically designed to evaluate alternative chemotherapy regimens in the elderly patient population. Alternative regimens studied have included weekly alternation of myelotoxic and non-myelotoxic agents, low toxicity anthracyline derivatives, or use of hemopoietic growth factors to support full doses of antiblastic agents. Despite the tolerance of these regimens being improved as a consequence of the short duration of therapy, the therapeutic results were identical to CHOP with no improvement in CR rate or FFP.

In one of the largest published series Zinzani *et al.* evaluated 350 patients aged over 60 years treated with a modified MACOP-B regimen called VNCOP-B in which less toxic drugs such as etoposide and mitoxantrone substituted other compounds (methotrexate and doxorubicin) of the same category or equally effective.[35] The overall response rate was 83% and the CR rate was 58% with no difference in FFP and OS at 69 months among three age-defined subgroups (60-69, 70-79, over 80 years). The results of a randomized study from the EORTC comparing CHOP to VMP (etoposide, mitoxantrone, and prednimustine) in NHL-patients older than 70 years have recently been reported, favoring CHOP in terms of response rate, two-year FFP and OS (77% vs 50%, 55% vs 22 %, 65% vs 30% respectively). Toxicity was similar but more patients in the VMP arm discontinued treatment because of progression.[36] More recently, the addition of rituximab to the backbone of the CHOP therapy regimen has been tested by the GELA group. Patients with B-DLCL were randomized to receive eight cycles of CHOP either alone or with the addition of rituximab. Preliminary data show that there is a statistically significant improvement in CR, FFP and OS in the arm receiving combined treatment.[37]

Although the maximum eligible age for HDT has been extended in selected patients in the last years, the majority of elderly patients are usually not candidates for transplantation programs and only a small number of studies on such patients have been published. In one of these Gopal *et al.* evaluated 53 patients aged over 60 years. Forty-four patients had aggressive histology, 75% had chemosensitive disease and all had failed anthracycline therapy. Conditioning regimens included busulfan, melphalan and thiotepa or cyclophosphamide, etoposide and total-body irradiation for the majority of patients. Estimated four-year overall survival and progression-free survival were 33% and 24%, respectively. The treatment-related mortality was 22%.[36]

In conclusion, age *per se* should not be considered as an absolute exclusion criterion for transplant programs.

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Aggressive Lymphomas
Chairmen: M. Baccarani, G. Torelli

Rituximab in combination with CHOP for the treatment of diffuse large B-cell lymphomas

BERTRAND COIFFIER
Hospices Civils de Lyon & Université Claude Bernard, Lyon, France

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Correspondence: Professeur Bertrand Coiffier, CH Lyon-Sud, 69495 Pierre Bénite, France. Phone: international +33.478.861194. Fax international +33.478.866566. E.mail bertrand.coiffier@chu-lyon.fr

Monoclonal antibodies have now been used for the treatment of lymphoma patients for 7 years, and were first developed for so-called *low-grade* or indolent lymphomas.[1] Although murine antibodies chelated with a toxin or a radioisotope have been used in clinical trials,[2-4] the majority of the results come from the use of rituximab, the first monoclonal antibody developed with activity by itself. This chimeric human-mouse antibody fixes the CD20 antigen on the membrane of lymphoma cells with the murine antibody part and stimulates the immune host mechanisms through the human Fc part. *In vitro* data have shown that rituximab may induce lymphoma cell lysis through activation of the complement cascade (complement-dependent cytolysis or CDC) and/or activation of immune cells through Fc fixation (antibody-dependent cell cytolysis or ADCC).[5,6] These immune mechanisms were originally thought to be only effective in low proliferating tumors, thus the first phase I and phase II studies only included patients with indolent lymphoma. These studies allowed the confirmation of the efficacy of this new type of anticancer drug.[1,7] Secondarily, other *in vitro* mechanisms of action were demonstrated such as induction of apoptosis, inhibition of cell proliferation, and synergism with standard chemotherapy drugs.[8,9] CD20 antigen is expressed on nearly all B-cells, except the early B-cell progenitors and the late plasmocytes. Therefore, recent years have seen the development of studies with monoclonal antibodies in other B-cell lymphoproliferative diseases, particularly diffuse large B-cell lymphoma and mantle cell lymphoma.

Diffuse large B-cell lymphoma (DLCL) is the most frequent lymphoma, representing >40% of all lymphomas. It may arise as a primary nodal or extranodal tumor but also as a transformation of an indolent lymphoma. It is not a homogeneous disease and several variants or subtypes have been described in the REAL and WHO classifications[10] but these variants and subtypes have not shown major differences in their response to treatment or outcome.[11] Usually, DLCL patients are treated according to their prognosis and CHOP chemotherapy is considered as the standard,[12] even if regimens with higher dosages of cyclophosphamide and doxorubicin or incorporating autologous stem cell transplantation have been associated with better results in patients with adverse prognostic parameters.[13,14] All DLCL express the CD20 antigen and may respond to therapy with monoclonal antibodies even if the proliferation rate is usually higher than in indolent lymphomas.

Approximately half of all patients diagnosed with aggressive non-Hodgkin's lymphoma (NHL) are older than 60 years and up to 80% of them have a DLCL.[11,15] The treatment of elderly DLCL patients poses a difficult clinical challenge. While the CHOP chemotherapy regimen is currently considered as the standard of care in younger patients, it has been described as excessively toxic in elderly patients.[16] Regimens specifically designed to be less toxic have been proposed for elderly patients,[17,18] but although these regimens caused fewer side effects they were also less effective, and, usually, provided a lower overall benefit than the CHOP regimen. Indeed, CHOP only induces a complete response in 40% of elderly DLCL patients, with a 3-year event-free survival rate of 30% and a 3-year overall survival rate of 35% to 40%.[18,19] Several new chemotherapy regimens have been developed in an attempt to improve on the results obtained with CHOP, generally by increasing the number of anticancer drugs administered, or by giving treatment at higher doses or more frequent intervals.[20] However, increasing the dosage or dose intensity of chemotherapy above the levels of standard CHOP is generally not as feasible in elder-

ly patients as in younger patients. Attempts to increase the efficacy of CHOP through the use of additional cytotoxic drugs may in fact require the use of decreased doses of the two major anti-lymphoma drugs, doxorubicin and cyclophosphamide, and, perhaps for this reason, the newer regimens have not been associated with better results in randomized trials.[12]

Phase II trial of rituximab in relapsing patients with DLCL

The first study evaluating the response rate in patients with aggressive lymphoma (DLCL or mantle cell lymphoma (MCL)) patients was conducted in Europe three years ago.[21] Patients were randomized between two doses of rituximab, either 375 mg/m^2 every week for 8 weeks or 375 mg/m^2 the first week followed by 500 mg/m^2 the following 7 weeks. Among the 54 patients included, five reached a CR and 12 a PR for an overall response rate of 32%, without difference between the two doses. The trial was not planned for observation of the duration of response but median duration of response was longer than 3 months at the time of publication. The observed toxicity was not worse than that described in patients with indolent lymphoma. Mostly grade 1 or grade 2 adverse events were observed, slightly more in patients treated with 500 mg/m^2 of rituximab. This study showed that more aggressive lymphomas than follicular lymphomas may respond to rituximab therapy and it opened up research on rituximab in all types of B-cell lymphomas.

Phase II studies combining rituximab and chemotherapy

One important phase II study has been presented on the combination of CHOP and rituximab in aggressive B-cell lymphomas.[22] In this study, 33 patients with previously untreated advanced aggressive B-cell NHL received an infusion of rituximab (375 mg/m^2) on day -2 of each cycle of CHOP chemotherapy for 6 cycles. The overall response rate was 94% (31 of 33 patients). Twenty patients reached a CR (61%), 11 patients a PR (33%), and 2 patients were classified as having progressive disease. The median duration of response and time to progression had not been reached after a median observation time of 26 months, 29 of the responding patients remaining in remission during this period, including 15 of 16 patients with an IPI score > 2. bcl-2 gene rearrangement was present in 39% of the patients, bearing witness to either a follicular large cell lymphoma or a transformation of follicular lymphoma, but no difference in response rate was observed for patients with or without bcl-2 rearrangement. Patients with a true DLCL or adverse prognostic parameters had a lower response rate. This study was not planned for survival observations but the duration of response seems longer than that usually observed with CHOP alone in this group of patients. The combination of CHOP and rituximab did not increase the toxicity of either type of chemotherapy. In this first report on the safety and efficacy of rituximab in combination with standard-dose CHOP for the treatment of aggressive B-cell lymphoma, the response rates seem higher than or at least comparable to those usually achieved with CHOP alone without significant added toxicity.

Randomized trials with rituximab plus chemotherapy

No study has been presented comparing the efficacy of rituximab alone to chemotherapy in DLCL patients. However, the GELA (*Groupe d'Etude des Lymphomes de l'Adulte*) has recently presented the preliminary results of a study in elderly patients with DLCL comparing 8 cycles of CHOP to 8 cycles of CHOP plus rituximab (R-CHOP).[23-25] The classical doses of CHOP were given every 3 weeks and rituximab was given at the dose of 375 mg/m^2 the same day as the CHOP (Figure 1). Administration of granulocyte colony-stimulating factor (G-CSF) was allowed if patients had febrile neutropenia or infection during the previous cycle and it was given to 50% of the patients. Four hundred newly diagnosed patients were included in this trial but only 328 have been included in the presented interim analysis, 159 in the CHOP arm and 169 in the R-CHOP arm. Patients were stratified for *Age-Adjusted International Prognostic Inde*x (IPI 26) scores (0-1 vs 2-3), had a performance status (PS) less or equal to 2, and no contra-indication to doxorubicin or vincristine. The primary endpoint was event-free survival (EFS), with events defined as disease progression or relapse, death, or initiation of new alternative treatment. Secondary endpoints were response rate, progression-free survival, disease-free survival, survival, and safety.

The median age of patients was 69 years, a little higher in the R-CHOP arm. Adverse prognostic parameters were equally distributed between arms: 63% of the patients had stage IV disease, 20% had PS >1, 38% had B symptoms, 65% had elevated lactate dehydrogenase (LDH), 27% had bone marrow involvement, 33% had bulky tumors, 52% had >1 extranodal disease sites, and 60% had an IPI equal to 2 or 3. At the time of the analysis, 98% of

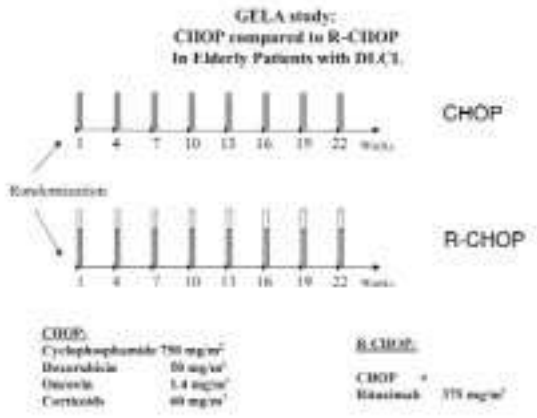

Figure 1. Design of the GELA study comparing CHOP to R-CHOP in elderly patients with diffuse large B-cell lymphoma.

cases were reviewed by an independent panel and DLCL histology was confirmed in 87%. Sixty-two patients withdrew early, 23 due to treatment failure (18 with CHOP, 5 with R-CHOP), 30 due to adverse events (17 with CHOP, 13 with R-CHOP), and 9 for other reasons.

Preliminary analysis revealed no major difference between the two arms in hematologic toxicity, or in grade 3 or 4 infection, mucositis, vomiting, liver, cardiac, neurologic, renal or lung toxicity. Seventeen patients (10%) had a grade 3 or 4 infusion-related syndrome during the first rituximab infusion. At the end of treatment, 76% of the patients had reached a CR or an undocumented CR (CRu) in the R-CHOP arm compared to 62% in the CHOP arm (p = 0.012). Twenty-two percent of the patients treated with CHOP had a progression during the treatment compared to 10% in the R-CHOP arm. With a median follow-up of 12 months, 77 events (48%) were observed in the CHOP arm and 49 (29%) in the R-CHOP arm, most of them being progression during or after treatment. Interim results based on data from all 328 patients (intent-to-treat analysis) are presented in Table 1.[23]

Table 1. Results of the interim analysis of 328 elderly patients with newly diagnosed DLCL and treated with CHOP or R-CHOP.[23]

	CHOP	*R-CHOP*	*p value*
Number of patients	159	169	-
CR/CRu	62%	76%	0.012
12-month event-free survival	49% (±8)	69% (±8)	< 0.0005
12-month overall survival	68% (±8)	83% (±7)	< 0.01

This study demonstrated that the addition of rituximab to CHOP chemotherapy led to significant prolongation of event-free survival (Figure 2) and overall survival in elderly patients with DLCL, without significant additional toxicity. Definitive results on the 400 randomized patients and with a longer follow-up will be presented before the end of 2001 but the magnitude of the difference between the two arms is so important that the probability of a modification of these results is very low.

Other randomized studies are ongoing in the same setting (elderly patients) or in younger patients with or without adverse prognostic parameters. If they confirm the results of the GELA study, the combination of CHOP plus rituximab will become the standard therapy for patients with diffuse large B-cell lymphoma, and probably all B-cell lymphomas.

In this study, rituximab was infused the same day as the CHOP regimen. In other studies, it was administered two days before each CHOP cycle, the same day as the CHOP, or interspersed before and in the middle of CHOP cycles. No clear rationale exists for these combinations. The preliminary trial used independent cycles of rituximab and CHOP because the putative toxicity of the combination was not known.[27] In the subsequent studies, rituximab was given two days before CHOP because analyses had shown that *in vitro* potentiation of anticancer drugs had required two days of incubation and because the corticosteroids given in the CHOP regimen may have decreased the immune effects of rituximab. However, another *in vitro* study showed that potentiation of doxorubicin did not need a preliminary incubation.[28] The GELA trial clearly demonstrates that rituximab may be given the same day as CHOP with a very good efficacy. Because of the long half-life of rituximab, the day of the infusion in combination with chemotherapy does not matter and doing both the same day is probably the easiest treatment for the patient.

Rituximab as maintenance therapy

Because the efficacy of rituximab seems greater when the tumor mass is smaller, its efficacy may theoretically be higher in patients responding to chemotherapy either as first line treatment or during relapse. While studies are ongoing in this setting for patients with follicular lymphoma or DLCL, none has yet been presented for DLCL patients.

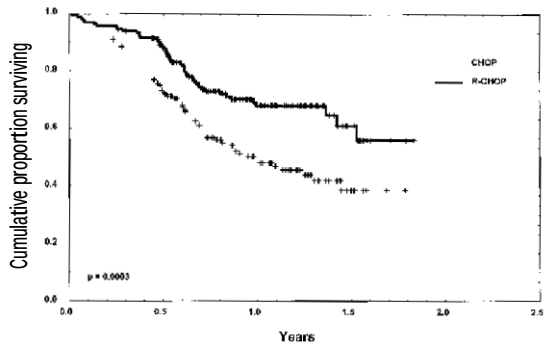

Figure 2. Event-free survival in the 328 patients of the randomized study comparing CHOP and R-CHOP in elderly patients with diffuse large B-cell lymphoma.

Moreover, because of the possible synergism between chemotherapy and rituximab, if both were given to a patient, the combination of the two would probably be better.

Rituximab before harvesting stem cells

In vivo purging with rituximab therapy has been shown to be effective in follicular lymphoma patients, most of them demonstrating the disappearance of bcl-2 rearranged cells in the harvest. No such marker exists in DLCL and the proportion of patients with circulating lymphoma cells is much less than in indolent lymphomas. Thus, the relevance of *in vivo* purging may be disputable and no study has been presented in such a setting.

In conclusion, these different studies show that rituximab has some activity by itself in DLCL and, more importantly, that this activity is increased when it is combined with CHOP. More studies will come and will allow the definition of the correct use of rituximab in combination with chemotherapy: which regimen? How many infusions of rituximab? Maintenance therapy? Currently, we can only recommend the combination we have used: 8 cycles of R-CHOP with rituximab given the same day as CHOP, every 3 weeks.

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Aggressive Lymphomas
Chairmen: M. Baccarani, G. Torelli

Oxaliplatin in aggressive lymphomas

IAN CHAU
Lymphoma Unit, Royal Marsden Hospital, Downs Road, Sutton, Surrey, United Kingdom

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Correspondence: Ian Chau, MD, Lymphoma Unit, Royal Marsden Hospital, Downs Road, Sutton, SM2 5PT Surrey, UK.

Although 35-50% of all intermediate and high grade non-Hodgkin's lymphoma (NHL) patients may be cured by anthracycline-containing chemotherapy, the remaining patients have poor long term outcome. High dose chemotherapy with autologous stem cell support is now an established consolidation treatment for chemosensitive relapse.[1] However not all patients are candidates for transplantation and it is important to have effective and well tolerated salvage therapy with minimal toxicities. One approach to improve outcome would be incorporation of novel chemotherapeutic agents with activity against lymphomas to improve existing combination regimens.

Platinum-containing regimens such as DHAP (dexamethasone, cytarabine and cisplatin),[2] ESHAP (etoposide, methyprednisolone, cytarabine and cisplatin)[3] and EPIC (etoposide, prednisolone, ifosfomide and cisplatin/carboplatin)[4] were developed in the 1980s to be non-cross-resistant to doxorubicin containing regimens used in front line therapy.

Long-term follow-up of platinum based salvage regimens from the M.D. Anderson Cancer Center showed a response rate of 41% for DHAP in 122 patients with relapsing or primary refractory NHL. Median survival was 6 months. The median time to treatment failure (TTF) was 0 because more than 50% of patients failed to achieve a response.[5] Seven early deaths were reported (3 from tumor lysis syndrome, 2 from sepsis, 1 from subdural hematoma and 1 from pulmonary thromboembolism). Twenty-eight patients (22%) had documented infection. Tumor lysis syndrome occurred in five patients and was fatal in three. Renal impairment with serum creatinine risen more than twice the baseline level occurred in 20% of patients and was permanent in 7% of patients. Severe peripheral neuropathy from cisplatin occurred in 4 patients and necessitated discontinuation of their treatment. Tinnitus was marked in 4 patients and severe hearing loss was noted in another three patients.[2]

In a subsequent study using ESHAP, 116 patients were evaluated. A response rate of 56% was reported. The median survival was 14 months and median time to treatment failure was 4.5 months.[5] Six patients experienced early deaths (5 from sepsis, 1 from congestive cardiac failure). Thirty per cent of patients developed febrile neutropenia. Again 22% of patients developed marked renal impairment with serum creatinine elevated to twice or more the baseline value and this was permanent in 4% of patients. Peripheral neuropathy was commonly observed in patients responding to ESHAP although the exact proportion of patients was not reported.[3]

Oxaliplatin is a new platinum compound that has proven activity in solid tumors such as primary cisplatin-resistant colorectal carcinoma[6,7] and secondary cisplatin/carboplatin-refractory ovarian carcinoma,[8-11] non-small cell lung cancer[12] and germ cell tumors.[13] In contrast to cisplatin, it has minimal renal or auditory toxicity.

Mechanism of action

Oxaliplatin is a third generation platinum compound with a 1,2-diaminocyclohexane (DACH) carrier ligand.[14] This important difference in the molecule, and hence in the DNA adducts formed, confers a different spectrum of activity compared with that of cisplatin.[15] The DACH platinum family of cytotoxic agents has been shown to have antitumoral activity in cell lines with intrinsic and acquired cisplatin resistance. Although the exact cytotoxic mechanism of oxaplatin is not known, computer models predict that the spatial DNA structural binding of non-leaving DACH adducts could play a role via its non-recognizability by DNA damage recognition proteins such as mismatch repair (MMR) complex.[16] The reason for limited cross-resistance between oxaliplatin and cisplatin/carboplatin has not been fully elucidated. The non-leaving DACH group, which is part of the

platinum-DNA adduct, induces specific conformational distortions, which lead to non-recognition by the MMR complex.

Pharmacokinetic studies

The pharmacokinetics (PKs) of unbound platinum in plasma ultrafiltrate after oxaliplatin administration are typically triphasic, characterized by a short initial distribution phase and a long terminal elimination phase.[17] No accumulation was observed in plasma ultrafiltrate after 130 mg/m^2 every 3 weeks or 85 mg/m^2 every 2 weeks. Interpatient and intrapatient variability in platinum exposure (AUC_{0-48}) was moderate to low (33% and 5%, respectively). Platinum bound irreversibly to plasma proteins and erythrocytes. Platinum was rapidly cleared from plasma ultrafiltrate at a rate that was similar to or exceeded the average human glomerular filtration rate (GFR). The renal clearance of platinum significantly correlated with GFR, indicating that renal filtration is a major mechanism of platinum clearance. Tissue distribution is an equally major mechanism of platinum elimination from the systemic circulation.

Clearance of ultrafilterable platinum was decreased in patients with moderate renal impairment; however, there was no increase in drug toxicity.[18] The effect of severe renal impairment on platinum clearance and toxicity is unknown. There was no significant effect of age, sex or moderate hepatic impairment on the clearance of ultrafilterable platinum.

Oxaliplatin undergoes rapid and extensive nonenzymatic biotransformation in plasma ultrafiltrate and urine.[19] There is no evidence of CYP 450-mediated metabolism. Oxaliplatin rapidly forms a variety of putative cytotoxic intermediates in blood and plasma, including the monochloro, dichloro and diaquo-DACH platin. Urinary elimination is the predominant route of platinum elimination, with fecal excretion accounting for only about 2% of the administered dose.

Safety profile

Oxaliplatin has been evaluated most extensively in colorectal cancer patients especially in combination with 5-fluorouracil ± leucovorin. The adverse events most often cited are hematologic, nausea, vomiting, diarrhea, mucositis, early onset cold-induced dysesthesia and a cumulative peripheral sensory neuropathy. Nephrotoxicity, cardiotoxicity and ototoxicity were not reported in clinical trials. Moreover, there is a very low incidence of alopecia.[20]

Hematologic side effects. In controlled trials, incidences of anemia were similar in both the oxaliplatin and control groups, suggesting that the condition is probably a manifestation of the underlying disease state. Neutropenia occurred more often in the oxaliplatin treated group especially in combination therapy. However, infection or febrile neutropenia was no more frequent in the oxaliplatin arm than in the control arm. Thrombocytopenia arises occasionally after multiple cycles of therapy.

Gastrointestinal side effects. Nausea and vomiting were readily controlled by administration of standard antiemetics such as 5HT3 receptor antagonists and/or dexamethasone. Grade 1-2 diarrhea was reported with oxaliplatin monotherapy, but more severe diarrhea has been reported with other cytotoxic drugs such as fluoropyrimidines.

Hepatotoxicity. Oxaliplatin has minimal impact on chemistry parameters of liver function. As discussed before, oxaliplatin is not metabolized in the liver to any extent. Therefore mild to moderate hepatic dysfunction does not warrant oxaliplatin dose modification or reduction.

Neurosensory syndrome. The symptoms reported can be separated into two distinct categories: a cold-induced dysesthesia with a rapid onset of hours to days following treatment and a late-onset cumulative sensory neuropathy observed after multiple cycles of therapy.

Acute onset dysesthesia is common, occurring in 80% to 85% of the patients, appears within hours of infusion, and is usually short-lived. Cold-contact provokes or exacerbates the characteristic acral and pharyngolaryngeal dysesthesias that are occasionally accompanied by muscular and laryngeal spasms. These events usually only occur during the first few days following infusion. No oxaliplatin dose reduction is required, but the infusion time may be prolonged from 2 to 6 hours. Patients should be instructed to avoid exposure to cold.

Dose-limiting peripheral sensory neuropathy occurs in 10 to 15% of patients after a total cumulative dose of 780 to 850 mg/m^2, usually after a response to treatment has been obtained. At first this dose-limiting toxicity presents as persistent paresthesia after multiple cycles of treatment. Clinical findings include decrease in vibration perception, proprioception, and fine point discrimination. Symptoms include difficulty in writing and impairment of fine manipulations such as buttoning a shirt. Multiple cycles of therapy may prolong the duration of these events. It is recommended that the oxaliplatin dose is decreased by 25% when paraesthesia becomes persistent between treatment cycles. The treatment needs to be stopped in cases of functional impairment. The cumulative

peripheral sensory neuropathy is reversible in the majority of the patients after discontinuation of treatment, with a median time to resolution of functional impairment of 15 weeks.

Clinical studies

As a single agent, oxaliplatin has been evaluated at a dose of 130 mg/m^2 every 3 weeks.[8;12;21-23] Grade 3 and 4 toxicities were infrequent in these studies. Oxaliplatin at the same dose schedule has also been tested in combination with 5-FU ± leucovorin,[24-26] raltitrexed,[27,28] and paclitaxel.[9] Acceptable toxicity profiles were again noted in these studies.

Oxaliplatin has been shown to be active in relapsed or refractory NHL.[29] It showed early promise as a single agent with a response rate of 40% in twenty-two patients with recurrent non-Hodgkin's lymphoma. One complete response and eight partial responses were observed. Oxaliplatin demonstrated moderate activity in different histologic subgroups in this study. Furthermore, one patient with cisplatin-refractory disease responded to oxaliplatin which suggested non-cross-resistance of oxaliplatin with cisplatin evident in other secondary cisplatin/carboplatin refractory tumors such as ovarian and germ cell cancers.[10,13] Duration of response was 27 months and the median survival was 23 months. However nearly two thirds of patients had an indolent histology which might have accounted for the long duration of response and median survival.

At our institution, a study was conducted to evaluate the activity and safety of oxaliplatin in combination with high dose Ara-C and dexamethasone (DHAX) substituting cisplatin with oxaliplatin in the DHAP regimen in patients with relapsed or refractory non-Hodgkin's lymphoma. Twenty-four evaluable patients with intermediate or high grade non-Hodgkin's lymphoma were treated with 3 weekly oxaliplatin (130 mg/m^2) on day 1, cytarabine (2 g/m^2 for 2 doses) on day 2 and dexamethasone (40mg) on days 1-4. The median age of patients was 58 (range=18-70). Histologic subtypes were: diffuse large B cell, 20; mantle cell 2; anaplastic large cell, 1; peripheral T cell, 1. The overall objective response rate (RR) was 50% (95% CI=29%-71%) including 4 complete responses and 8 partial responses. RR for those patients treated at first relapse was higher than those treated at second and subsequent relapse (77% vs. 29%). Grade 3 and 4 toxicity was mainly hematologic anemia 17%, neutropenia 75% and thrombocytopenia 75%. No grade 4 non-hematologic toxicity was reported. No significant renal or neurotoxicity was demonstrated. Median survival was 10.6 months. Probabilities of 1-year progression free survival and overall survival were 47% (95% CI = 26-66%) and 50% (95% CI = 23-72%), respectively.

Conclusions

Oxaliplatin is a new platinum compound that has significant activity in solid tumors. Although its experience in anti-lymphoma therapy is still limited, it has significant activity with an acceptable toxicity profile. Lack of renal and neurotoxicity would make oxaliplatin an attractive alternative to cisplatin, especially as part of a cytoreductive regimen before high dose chemotherapy and stem cell transplantation.

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Aggressive Lymphomas
Chairmen: M. Baccarani, G. Torelli

Radioimmunotherapy with 90-yttrium Zevalin for indolent or aggressive non-Hodgkin's lymphomas

ANTONIO J. GRILLO-LÓPEZ
IDEC Pharmaceuticals Corporation, San Diego, CA, USA

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Correspondence: A.J. Grillo-López, IDEC Pharmaceuticals Corporation, San Diego, California 92121, USA. E-mail: agrillo@idecpharm.com

Yttrium-[90] ibritumomab tiuxetan (^{90}Y Zevalin™) is composed of ibritumomab covalently bound to tiuxetan and stably chelated to the radionuclide, ^{90}Y. Yttrium-[90] was chosen in preference over iodine-[131] because of its superior features as shown in Table 1.

The radionuclide, indium-[111] (^{111}In), has a similar half-life and biodistribution when substituted for ^{90}Y in the ibritumomab tiuxetan conjugate and is used as a surrogate for imaging and dosimetry, if desired.

A course of ^{90}Y ibritumomab tiuxetan outpatient treatment consists of an initial infusion of rituximab to deplete B-cells from the peripheral circulation, bone marrow, and lymph nodes, and to optimize ^{90}Y ibritumomab tiuxetan biodistribution. One week later, patients receive a second infusion of rituximab followed by a single injection of ^{90}Y ibritumomab tiuxetan. The standard ^{90}Y ibritumomab tiuxetan dose, 0.4 mCi/kg body weight (15 MBq/kg; not to exceed 32 mCi [1.2 GBq]), is reduced to 0.3 mCi/kg (11 MBq/kg) in patients with mild thrombocytopenia (100,000 to 149,000 platelets/mm^3). Imaging and dosimetry are not necessary, but if desired, an imaging dose of ^{111}In ibritumomab tiuxetan (5 mCi [185 MBq]; 1.6 mg) may be infused following the initial rituximab infusion.

Clinical development

The clinical development of ^{90}Y ibritumomab tiuxetan was initiated in 1993. Seven clinical trials (6 complete and 1 ongoing) have been performed (Table 2).

Phase III comparison study (106-04)

A phase III, randomized, controlled clinical study was designed to compare the radioimmunotherapy ^{90}Y ibritumomab tiuxetan (N=73) with the bioimmunotherapy rituximab (N=70) in patients with low-grade, follicular, or transformed non-Hodgkin's lymphoma (NHL). The prospectively defined primary endpoint was overall response rate (ORR) as determined by LEXCOR, a group of experts in radiology and oncology. LEXCOR was blinded to the treatment received and to the investigator's response assessment (Table 3). Secondary endpoints included time to progression (TTP), duration of response (DR), time to next anticancer therapy (TTNT), and quality of life assessment (QOL). At enrollment, patients were stratified by histology (IWF A versus follicular versus transformed) and were randomized to receive ^{90}Y ibritumomab tiuxetan or rituximab.

No statistically or clinically relevant differences were found at study entry between the ^{90}Y ibritumomab tiuxetan treatment group and the rituximab treatment group.

The overall response rate for Zevalin was 73% as compared to 47% for rituximab (p= 0.002). By International Workshop criteria the overall response rate to Zevalin was 80%. Zevalin produced not only a higher response rate but also a larger decrease in overall tumor volume as compared to rituximab. The tumor shrinkage, as measured by the percent decrease in SPD (sum of the products of the perpendicular diameters of all measurable lesions), was –90.9% for Zevalin and –70.5% for rituximab (Table 4).

Chemoresistance, defined as resistance to any chemotherapy or resistance to last chemotherapy, did not result in any significant decrease in response rate to Zevalin as shown in Table 5.

As shown in Table 6, duration of response and time to progression were not significantly different for Zevalin versus rituximab. The study, however, was not powered to show a difference but

Table 1. Features of yttrium-[90] compared with iodine-[131].

Properties	*Yttrium-[90]*	*Iodine-[131]*
Energy	Beta emitter (2.3 MeV)	γ (0.36 MeV)/β (0.6 MeV) emitter
Path Length	χ_{90} 5 mm	χ_{90} 0.8 mm
Administration	Outpatient	Inpatient or with restrictions to protect family/environment
Half-Life	64 Hours	192 Hours
Urinary Excretion	Minimal, 7% in 7 Days	Extensive/variable, 46% to 90% in 2 days
Dosing	Dose based on weight and baseline platelet count	Tracer dose and dosimetry used to customize dose

Table 2. ^{90}Y ibritumomab tiuxetan clinical studies.

Study	*Description*	*N*	*Status*
106-01/02	Phase I/II	18	Complete
106-03	Phase I/II	58	Complete
106-04	Phase III Controlled Randomized 0.4 mCi/kg	143	Complete
106-05	Phase II Mild Thrombocytopenia 0.3 mCi/kg	30	Complete
106-06	Phase III Rituximab-Refractory Non-randomized 0.4 mCi/kg	57	Complete
106-98	Phase II Open Label	138	Ongoing
	Total	444	

N = number of patients.

Table 4. Change in median SPD: phase III comparison study.

Treatment Group	*N*	*Median Baseline SPD (cm²)*	*Median % Change in SPD**	*p†*
^{90}Y Ibritumomab Tiuxetan	73	21.4	-90.9	0.004
Rituximab	69‡	25.0	-70.5	

†p-value generated by Wilcoxon rank sum test; ‡lesion-measurement data for one patient was not available.

Table 5. Response in chemotherapy-resistant* patients: Phase III comparison study.

	Response	*^{90}Y Ibritumomab Tiuxetan (N = 73) N (%)*	*Rituximab (N = 70) N (%)*	*p p-value†*
Resistance To Any Chemotherapy	CR, CCR, or PR	24 (63)	18 (43)	0.078
	SD or PD	14 (37)	24 (57)	
Resistance To Last Chemotherapy	CR, CCR, or PR	21 (64)	11 (36)	0.045
	SD or PD	12 (36)	20 (65)	

**Chemotherapy-resistant: non-responders or progressed within 6 months; †p-value generated by Fisher's exact two-tailed test.*

Table 3. Overall response rates: phase III comparison study.

	Protocol-Defined Response Criteria			*International Workshop Response Criteria*		
	90Y Ibritumomab Tiuxetan	*Rituximab*	*p-value**	*^{90}Y Ibritumomab Tiuxetan*	*Rituximab*	*p-value**
ORR	73%	47%	0.002	80%	56%	0.002
CR	18%	11%	0.326	30%	16%	0.040
CCR/CRu	3%	4%	–	4%	4%	-
PR	52%	31%	–	45%	36%	-

**p-values generated by Cochran-Mantel-Haenszel test adjusted for histology type.*

Table 6. Summary of duration of response* in months for responders phase III comparison Study (N = 143).

Histology Type		*^{90}Y Ibritumomab Tiuxetan (n = 53)*	*Rituximab (n = 33)*	*p value*
All	n	53	33	0.644†
	Median	14.2+	12.1+	
	Range	(0.9, 28.9+)	(2.1, 24.5)	
	95% CI	[9.4, .]	[8.0, 24.5]	
	% Censored	47.2%	42.4%	
A	n	6	3	0.420‡
	Median	9.8	.	
	Range	(5.0, 20.5)	(8.0, 14.5+)	
	95% CI	[7.1, 20.5]	[8.0, .]	
	% Censored	16.7%	66.7%	
Follicular	n	42	27	0.371‡
	Median	18.5+	12.1+	
	Range	(1.7, 28.9+)	(2.7, 24.5)	
	95% CI	[10.0, .]	[7.9, 24.5]	
	% Censored	52.4%	40.7%	
Transformed	n	5	3	0.850‡
	Median	6.8	11.7	
	Range	(0.9, 20.3+)	(2.1, 17.0+)	
	95% CI	[0.9, .]	[2.1, .]	
	% Censored	40.0%	33.3%	

†p-value generated by proportional hazard regression adjusted for histology type; ‡p-values generated by logrank test.

Table 7. Time to progression* in months for ITT patients (N = 143).

Histology Type		*^{90}Y Ibritumomab Tiuxetan (n = 53)*	*Rituximab (n = 33)*	*p value*
All	n	73	70	0.173‡
	Median	11.2+	10.1+	
	Range	(0.8, 31.5+)	(0.7, 26.1)	
	95% CI	[7.8, 15.4]	[6.8, 12.9]	
	% Censored	37.0%	28.6%	
A	n	9	8	0.767‡
	Median	8.4	8.3	
	Range	(2.1, 21.7)	(1.0, 16.1+)	
	95% CI	[6.3, 12.1]	[1.7, .]	
	% Censored	11.1%	37.5%	
Follicular	n	55	58	0.062‡
	Median	12.6+	10.2+	
	Range	(2.9, 31.5+)	(0.7, 26.1)	
	95% CI	[9.3, 19.9]	[6.9, 13.1]	
	% Censored	43.6%	27.6%	
Transformed	n	9	4	0.576‡
	Median	3.1	10.1	
	Range	(0.8, 21.7+)	(0.7, 18.7+)	
	95% CI	[2.1, 8.0]	[0.7, .]	
	% Censored	22.2%	25.0%	

†p-value generated by proportional hazard regression adjusted for histology type; ‡p-values generated by logrank test.

Table 8. Severity and duration of hematologic toxicity.

Toxicity	*Incidence of grade 3 toxicity N (%)*	*Incidence of grade 4 toxicity N (%)*	*Duration* all patients (Days)*	*Duration* patients with grade 3 or 4 (Days)*
Overall Safety Analysis N = 349				
Neutropenia	103 (29.5)	105 (30.1)	15	23
Thrombocytopenia	185 (53.0)	35 (10.0)	17	28
Anemia	46 (13.2)	14 (4.0)	0	14

**Median duration of grade 3 or 4 toxicity.*

was designed to show a clinically meaningful advantage for Zevalin. All the Kaplan Meier graphs showed an advantage for the Zevalin arm of the study.

Toxicity was primarily hematologic (Table 8). In the 349 patient safety database, there was a 60% incidence of grade 3 and 4 neutropenia, 63% thrombocytopenia, and 17% anemia. Duration of grade 3 and 4 toxicity was 28 days for thrombocytopenia, 23 days for neutropenia, and 14 days for anemia. These timeframes are two weeks longer due to the conservative manner in which they were measured. If one measures the durations from the first count showing grade 3 or 4 to the last count showing grade 3 or 4, the durations would be up to two weeks shorter. The incidence of grade 3 or 4 infection or hospitalization was less than 7%.

Efficacy summary

In the phase III comparison study, the ORR was significantly higher in the ^{90}Y ibritumomab tiuxetan treatment arm (PDRC: 73% versus 47%, p = 0.002; IWRC: 80% versus 56%, p = 0.002) and the estimated median TTP was nearly equivalent between the treatment groups (11.2+ months for ^{90}Y ibritumomab tiuxetan patients; 10.1+ months for rituximab patients). Kaplan-Meier curves are consistent, with a longer median TTP in follicular, and possibly ITT, patient populations. The TTNT

Kaplan-Meier curves demonstrate that ^{90}Y ibritumomab tiuxetan-treated non-transformed patients have a longer time off therapy than do rituximab control patients.

In the phase III rituximab-refractory study, ^{90}Y ibritumomab tiuxetan induced responses in the majority of patients (PDRC: 59%; IWRC: 74%) with a significantly longer DR compared with prior rituximab therapy. In this relapsed and refractory low-grade, follicular, or transformed incurable NHL population, ^{90}Y ibritumomab tiuxetan therapy resulted in a higher ORR and longer treatment-free interval.

Safety summary

In the overall safety population, adverse events were primarily hematologic, transient, and reversible. Grade 4 neutropenia, thrombocytopenia, and anemia occurred in 30%, 10% and 4% of patients, respectively.

- Most non-hematologic adverse events were grade 1 and 2 and were related to accompanying rituximab infusions
- No major acute organ dysfunction
- Median serum immunoglobulins remained largely within the normal range despite a 6-month reversible depletion of B-cells
- 1.4% incidence of HAMA/HACA
- Less than 7% incidence of febrile neutropenia or infection requiring hospitalization
- No observable age-dependent differences in the safety profile

Rare cases of MDS were well within the expected background rate for this heavily pretreated patient population.

Dosimetry summary

Dosimetry was conducted in a total of 179 patients across four ^{90}Y ibritumomab tiuxetan studies and produced normal organ and red marrow radiation absorbed dose estimates well within the protocol-defined safety limits of 2000 cGy for normal organs and 300 cGy for red marrow. Dosimetric and pharmacokinetic parameters including estimated red marrow radiation absorbed dose, total body dose, effective half-life, and AUC do not correlate with hematologic toxicity. Dosimetry has been safely eliminated from the ^{90}Y ibritumomab tiuxetan regimen.

Conclusions

The ^{90}Y ibritumomab tiuxetan treatment regimen is completed in two outpatient appointments spaced one week apart, with no requirement for hospitalization, isolation, or shielding, due to lack of gamma emissions from ^{90}Y. The high energy and long pathlength of the pure beta emission from ^{90}Y allow effective treatment of bulky or poorly vascularized tumors. The specific targeting of tumor cells allows systemic therapy without side effects of hair loss, nephrotoxicity, or neurotoxicity. The response rate in chemotherapy-resistant patients is noteworthy. Time to next therapy (time off therapy) is prolonged.

Ibritumomab tiuxetan therapy represents a clinically meaningful advance in therapy for patients with relapsed or refractory, low-grade, follicular, or transformed B-cell NHL.

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Indolent Lymphomas
Chairmen: E. Morra, P. Rossi Ferrini

Indolent lymphomas: a therapeutic update

A. HAGENBEEK
Department of Hematology, University Medical Center Utrecht, The Netherlands

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Correspondence: A. Hagenbeek, Department of Hematology, University Medical Center Utrecht, The Netherlands.
E-mail: a.hagenbeek@lab.azu.nl

The treatment of patients with disseminated follicular non-Hodgkin's lymphoma (NHL) stages III and IV is a major challenge in hemato-oncology today. Despite the exploration of numerous treatment modalities during the past 50 years, the median survival time (7-9 years) has not really changed. In other words, the natural behavior of this group of indolent diseases has not been influenced significantly.

What has been achieved so far?

With single agent chemotherapy (chlorambucil, cyclophosphamide and more recently the purine analogs, in particular fludarabine), irradiation (total lymph node irradiation, low dose total body irradiation) and combination chemotherapy (varying from mild combinations such as COP to more intense regimens such as CHOP, fludarabine-based regimens - FND- and ProMACE-MOPP) a remission can be induced in 50-85% of patients. However, in general in comparative trials these various treatment approaches have not shown any particular regimen or modality which has led to a superior progression-free survival (PFS) and certainly not to an improved overall survival (OS). Whether remission is established early after diagnosis or after a *wait-and-see* period does not seem to play a role in terms of the duration of either PFS or OS.

Apparently, in the vast majority of patients who reach a (complete) remission after initial treatment a significant number of viable clonogenic NHL cells survive, which cause a relapse later on. It seems likely that at diagnosis NHL cells are present that are resistant to conventional chemo-radiotherapy. Although second, third, fourth, etc. remissions can be induced subsequently, the proportion of resistant cells increases step by step, leading to progressively shortened intervals between time to progression or time to next treatment. Finally, the patient ends up with refractory disease which limits the OS. Based on the above, important issues for further (pre)clinical research should deal with unravelling the mechanisms of drug resistance and translating these findings into new treatment approaches. It is envisaged that, as in the leukemias, small molecules will be developed that specifically interfere with the bcl-2 driven strong anti-apoptotic pathway.

In the 1980s a new player entered the field, i.e. interferon. This biological response modifier, in particular in conjunction with chemotherapy, appeared to prolong PFS and even — for the first time — an increase in OS was reported. The interferon seems to be most effective combined with anthracycline-containing regimens in patients with poor prognosis. In addition, some positive data concerning interferons suggest a dose-effect relationship. Despite these encouraging results, it must be concluded that interferons have so far not been included in the daily practice of follicular NHL treatment (side-effects?, costs?).

Recent developments and current questions to be answered from ongoing studies

As regards the development and introduction of new treatment modalities for follicular NHL, the past 10 years can be characterized as *never a dull moment*.

Marrow-ablative treatment followed by stem cell transplantation was introduced. Numerous phase II clinical trials on the role of autologous stem cell transplantation with or without graft purging have been published. However, in 2001 it is still not clear what the role of autologous stem cell transplantation is in the treatment of follicular NHL in first or subsequent remission. Only a few prospective randomized phase III, clinical trials were initiated and they either failed because of a lack of patient accrual (patients' and doctors' reluctance; fear of late

side-effects, such as secondary MDS/AML) or the follow-up is at present too short to permit any conclusions. The expectation is that autologous stem cell transplantation may well prolong PFS but will most probably not improve OS. Here again, resistant NHL cells survive even high-dose chemo-radiotherapy. This indicates that the limits of the possible with chemotherapy or radiotherapy have been reached. *The higher the dose, the better* does not seem to hold for follicular NHL. This conclusion makes it clear that completely new treatment modalities are needed to eradicate resistant NHL cells.

And that is where effective immunotherapy has come in most recently. On a small scale T-cell mediated immunotherapy has been found to be effective. Firstly, the graft-versus-leukemia effect after allogeneic stem cell transplantation in follicular NHL has clearly been established. There is a growing number of long term disease-free survivors of whom a fraction appear to be cured. The most direct evidence for allo-reactive T-cells eradicating NHL comes from clinical observations on the efficacy of donor lymphocyte infusions in patients who have relapsed after allogeneic stem cell transplantation. However, the drawback with this approach is treatment-related morbidity and mortality (toxicity of high-dose treatment, concomitant graft-versus-host disease and severe infections). If this approach can be translated into safer procedures (reduced-intensity conditioning, manipulating graft-versus-host/graft-versus-lymphoma by suicide gene therapy, development of T-cell clones specifically directed towards NHL, etc.), T-cell mediated immunotherapy of follicular NHL will become even more promising. Secondly, positive results have been reported from vaccination studies employing NHL DNA or idiotype protein. Further results from ongoing (randomized) clinical trials will reveal what role vaccination plays in follicular NHL.

Finally, undoubtedly the most exciting recent data were generated from studies employing passive immunotherapy with monoclonal antibodies. After the early era in which murine monoclonal antibodies were used with disappointing results due to formation of human anti-mouse antibodies, the breakthrough came from the introduction of genetic engineering which allowed the creation of chimeric antibodies or even humanized/human antibodies, all with decreased immnunogenicity. So far, the CD20 antigen is probably the ideal target for B-cell NHL, as this antigen is lacking on normal stem cells or precursor B-cells, but is found on normal mature B-cells and B-NHL cells. In addition CD20 is not shed or modulated. With the chimeric antibody MabThera (rituximab) it appeared to be possible to induce complete (or even molecular) remissions in patients in relapse or refractory to chemotherapy after heavy pretreatment. One of the most attractive features of this approach is its favorable toxicity profile. The first data on the combination of MabThera treatment with conventional chemotherapy, interferon or autologous stem cell transplantation (*in vivo* purging) are promising, but results from ongoing randomized trials will determine whether these combinations improve treatment outcome.

When a radioisotope is coupled to the monoclonal antibody, treatment becomes even more effective. So far, there is ample experience with both ^{90}yttrium and ^{131}iodine coupled to a murine anti-CD20 monoclonal antibody (Zevalin and Bexxar, respectively). The advantage of this combined modality treatment is two-fold: it is independent of the recruitment of patient's immune effector mechanisms and the radiation is capable of killing cells from a distance of several cell diameters. Thus, antigen-negative NHL cells which may be present are also killed. Obviously, more toxicity is encountered; in particular hematologic toxicity increases with increasing infiltration of the bone marrow by NHL cells. However, these toxic side-effects are brief and manageable.

Challenges for the future

Given the recently emerged new tools against follicular NHL, the major challenge for the future is how to create the most effective combinations and in which order. Tumor load reduction followed by T-cell mediated immunotherapy or radioimmunotherapy with monoclonal antibodies is an attractive policy with the aim of eradicating minimal residual disease. Studies are ongoing. It now appears possible to *surprise* the follicular NHL cells by subsequent attacks with agents employing completely different modes of action. For the first time there are prospects for significant prolongation of OS and even cures.

Finally, if the prognosis of an individual patient at diagnosis can be defined better by both clinical and biological parameters, it is envisaged that patient-tailored treatment will be introduced, aiming at the highest possible efficacy in conjunction with justifiable toxicity. At last, the natural behavior of follicular NHL will no longer be able to withstand intelligent intervention from the outside.

Indolent Lymphomas
Chairmen: E. Morra, P. Rossi Ferrini

Gemcitabine in lymphomas

PIER LUIGI ZINZANI, MONICA TANI, VITTORIO STEFONI, PATRIZIA ALBERTINI, LAPO ALINARI, ERNESTO VIGNA
*Institute of Hematology and Medical Oncology "L. e A. Seràgnoli", University of Bologna, Italy

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Correspondence: Pier Luigi Zinzani, MD, Institute of Hematology and Medical Oncology "L. e A. Seràgnoli, University of Bologna, Italy.
E-mail: plzinzo@med.unibo.it

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is an analog of deoxycytidine. It is transformed to the active triphosphate (dFdCTP) after intracellular phosphorylation. Gemcitabine monophosphate is inserted into the DNA and inhibits DNA elongation as a false nucleotide. In contrast to other antimetabolites, an additional, altered nucleotide is inserted behind dFdC inhibiting repair mechanisms (masked chain termination).

By this, *repair enzymes* (exonucleases) of the DNA are inhibited and repair mechanisms are prevented. This factor, as well as enzymic inhibition of gemcitabine diphosphate, lead to high intracellular concentrations of gemcitabine and enforce the cytostatic effect. Gemcitabine is mostly inserted into DNA but partly also into RNA. Competition at the receptor with the nucleoside deoxycytidine phosphate (dCTP) leads to a competitive inhibition of DNA polymerases.[1]

The effects of gemcitabine on cellular metabolism therefore include:

- inhibition of ribonucleotide reductase with lowering/disruption of deoxynucleotide *de novo* synthesis (mostly dCTP);
- multiplication of effect of deoxycitidine kinase by inhibition of the negative feedback on this enzyme, leading to enhanced phosphorylation of gemcitabine;
- inhibition of the enzyme responsible for the elimination of gemcitabine (deoxycitidine monophosphate deaminase). By exhausting the dCTP pool and increasing the intracellular concentration of dFdCTP, the positive feedback mechanism of the enzyme is further inhibited;
- inhibition of cytidine triphosphate synthetase (CTP-synthetase) leading to a further exhaustion of the dCTP pool and inhibition of RNA synthesis.

Pharmacokinetics

After infusion of 1,000 mg/m^2 of gemcitabine over 30 min, maximal concentrations of 10 to 40 µg/mL are observed. The extracellular half-life is approximately 30 minutes. Gemcitabine is metabolized to the cytostatically inactive metabolite 2'desoxy-2',2'-difluorouridine (dFdU) at a rate of 91-98%. Metabolization occurs in liver, kidneys, blood and other tissues via cytidine deaminases. After infusion of 1,000 mg/m^2, 92-98% of the dose is recovered in urine within one week. The excretion of the original substance and dFdU via the urinary tract is 99% with less than 1% being eliminated in the feces. Cytostatically active metabolites of gemcitabine are not detectable in plasma or urine. The plasma protein binding of gemcitabine is only 10%.

So far, gemcitabine has been found to demonstrate a broad spectrum of activity in solid tumors, including pancreatic, ovarian, breast, lung and bladder cancers. In hematopoietic malignancies, gemcitabine has shown a high level of activity as a single agent in relapsed or refractory Hodgkin's disease and some degree of efficacy in aggressive and indolent non-Hodgkin's lymphoma.

Hodgkin's disease (HD)

The study reported by Santoro *et al.*[2] was the first to evaluate the activity of gemcitabine in patients with previously treated HD. The drug dose was 1,250 mg/m^2 intravenous infusion on days 1, 8, and 15 of each 28-day cycle of therapy. The incidences of complete and partial responses were promising and strongly suggested a possible role for this drug in the management of HD. Of the 22 patients, two patients (9%) reached a state of complete remission, and seven patients (30%) achieved a partial response, for an overall response rate of 39%. The likelihood of achieving a response was not influenced by the patients' main pretreatment charac-

teristics or by their response to their last prior chemotherapy. The median duration of response was 7 months, and the median overall survival time was 11 months. In addition, in our experience[3] of 14 pretreated patients we obtained an overall response rate of 43% with 2 (14%) patients who achieved complete remission and 4 (29%) patients who had a partial response. In particular, both patients who had relapsed after autologous bone marrow transplantation achieved a response. The gemcitabine dose was 1,200 mg/m^2 with the same schedule as that used by Santoro. Another positive experience was reported by Lucas *et al*.[4]

Recently, the *German Hodgkin Lymphoma Study Group* started a study developing a BEACOPP variant (BAGCOPP) with the intention to reduce the rate of acute and long-term toxicities (particularly secondary malignancies) while maintaining or improving the regimen's efficacy by replacing etoposide with gemcitabine. The primary objective of the phase I part of the study is the determination of the maximum tolerated dose (MTD) of gemcitabine with regard to the timely and adequately dosed application of the therapeutic regimen. Three patients will be included at each dose level consecutively (800, 1000, 1250, 1500, 1750 mg/m^2 etc, at days 1 and 4, respectively). After reaching dose limiting toxicities and the determination of the MTD of gemcitabine, the phase II part of the BAGCOPP study will start. About the rationale for the treatment schedule, current information concerning gemcitabine comes from studies in solid tumors and lymphomas in which single doses of 750 to 2,500 mg/m^2 have been investigated with infusions lasting between 30 and 70 minutes. The maximum dose used was 4,560 mg/m^2. Almost all protocols included three single doses one week apart from each other followed by a one-week rest period. Only a few studies tested multiple infusions within one week or at longer intervals. The hematologic substances cyclophosphamide, adriamycin and etoposide will be given at the start of each cycle (days 1 to 3) in the BEACOPP protocol in order to allow adequate stem cell recovery. Two single administrations of gemcitabine on days 1 and 4 are planned in the BAGCOPP protocol with the aim of adhering to this principle. In the light of pharmacokinetic data, three days of rest between two gemcitabine administrations are regarded feasible.

Aggressive non-Hodgkin's lymphoma (NHL)

A multicenter phase II trial was conducted by Fossa *et al*.[5] in patients with relapsed or refractory aggressive NHL. Thirty patients with B-cell intermediate or high-grade NHL were enrolled into the study. No complete responses were observed, but six patients showed a partial response, 11 stable disease, and 13 progressive disease. The overall response rate was 20%. The median duration of partial response was 6 months. All patients who resonded had a histologic diagnosis of diffuse large-cell lymphoma whereas no patient with a diagnosis of intermediate- or high-grade lymphoma other than diffuse large-cell lymphoma showed a response. No statistically significant association with response rates was found for any clinical parameter. Notably, three patients with a complete response and three patients with no response after the last combination chemotherapy responded to gemcitabine. This study demonstrated the moderate efficacy of gemcitabine in the setting of relapsed or refractory aggressive lymphoma. It is difficult to compare these response rates to results reported in phase II trials using other drugs as single agents in patients with relapsed or refractory NHL. Furthermore, independent confirmatory studies evaluating the same drug are frequently missing. Considering these difficulties, the observed efficacy and toxicity of gemcitabine compare well with results of studies using single agents that are frequently incorporated in combination chemotherapy regimens for treatment of aggressive NHL, such as etoposide, mitoxantrone, or cisplatinum. The results also seem interesting when compared with those from other novel chemotherapeutic agents under investigation in NHL, such as paclitaxel or the topoisomerase I inhibitors CPT-11.

Other experiences have been described in aggressive NHL. Particularly, Savage *et al*.[6] reported that gemcitabine showed substantial activity in heavily pretreated middle-aged to elderly patients with advanced aggressive NHL. In this study the maximum dose of gemcitabine was 1000 mg/m^2 given weekly and the drug was infused, as in previous studies, over 30 minutes. Based on pharmacokinetic data, it is possible that a prolonged infusion might be more effective and less toxic.[7,8] On this basis, in a new ongoing phase II study Savage *et al.* have increased the infusion time from 30 to 180 minutes.

Bernell and Ohm also reported responses in two out of three patients with aggressive lymphoma treated with a dose of 800 mg/m^2/week.[9]

Indolent NHL

The study reported by Dumontet *et al*.[10] represented the first trial evaluating the efficacy of gemcitabine as a single agent in patients with

relapsed or refractory indolent lymphomas. Thirty-five patients were enrolled into the study, including 11 cases of mantle cell lymphoma (MCL), 10 cases of chronic lymphocytic leukemia (CLL)/lymphocytic lymphoma, nine cases of follicular lymphoma, four cases of lymphoplasmacytic lymphoma and two cases of T-cell lymphoma. Gemcitabine 1000 mg/m^2 was administered as a 30-min infusion on days 1, 8 and 15 of a 28-day schedule, up to a maximum of six cycles. Complete responses were observed in two patients with MCL, and partial responses were observed in seven patients, including three patients with CLL/lymphocytic lymphoma, two patients with T-cell lymphoma, one patient with MCL and one patient with follicular lymphoma. The overall response rate for the entire patient population was 25%. In addition, minor responses were observed in three patients, including two patients with MCL, and one patient with CLL. The median duration of response was 150 days and the overall progression-free survival was 342 days. There was a trend towards longer progression-free survival in patients who had not received prior nucleoside analog therapy than in patients who had received such therapy.

Preclinical data had shown significant *in vitro* efficacy on B-CLL cells and myeloma cell lines.[11,12]

T-cell disorders

Concerning cutaneous T-cell lymphoma (CTCL), we conducted a phase II trial in 44 consecutive, previously treated patients with mycosis fungoides (MF) (30 cases) and peripheral T-cell lymphoma unspecified (PTCLU) (14 cases) with exclusive skin involvement.[13] Gemcitabine was given to all patients on days 1, 8, and 15 of a 28-day schedule at a dose of 1,200 mg/m^2 for a total of three cycles. Of the 44 patients, five (11.5%) achieved complete responses, 26 (59%) partial responses, and the remaining 13 showed no benefit from the treatment. Two of the complete responses were histologically confirmed. The complete and partial response rates were the same for patients with MF and those with PTCLU, respectively. No difference in terms of overall response rate was observed between relapsed and refractory patients. The median durations of complete response and partial response were 15 months and 10 months, respectively. This report has confirmed our preliminary data on 13 patients,[14] who are also included here with a longer follow-up; in this study, gemcitabine-treated patients had a higher or at least comparable overall response rate compared with literature data on patients with MF treated with other nucleoside analogs, such as fludarabine and pentostatin.

Recently, we started a phase IIb multicenter study with gemcitabine as primary chemotherapy of patients with advanced CTCL (or pretreated only with PUVA or radiotherapy). The patients will be recruited from the *Italian Cutaneous Lymphoma Study Group*.

Sallah *et al*.[15] reported their experience in 10 patients with refractory and relapsed T-cell malignancies treated with gemcitabine. Two patients had CTCL, 2 prolymphocytic leukemia (PLL), 2 nodal PTCL, 2 small lymphocytic lymphoma (SLL), 1 anaplastic and 1 angiocentric lymphoma. The drug dose was the conventional 1,200 mg/m^2 on days 1, 8 and 15 of each 28-day cycle. Of the 10 patients, two achieved a complete response (1 PLL and 1 anaplastic) and four a partial response (2 CTCL, 1 angiocentric, 1 PTCL) for an overall response rate of 60%. The median and mean duration of response was 13 and 16 months, respectively.

Toxicity

Hematologic toxicities: anemia of WHO grade III was observed in 5-10% of patients, neutropenia of WHO grades III and IV in 20% and 10% of patients, respectively, WHO grade III and IV thrombocytopenia in 20% and 10% of patients, respectively.

Non-hematologic toxicity: transient elevations in liver transaminases were observed in 5-10% of patients. Renal and pulmonary toxicity was very rare; WHO grade III-IV less than 1%. Flu-like symptoms with headache, fever, myalgias and fatigue occurred in up to 10% of patients. No alopecia usually occurs during gemcitabine therapy. Neurotoxicity in connection with gemcitabine is rare. Peripheral edema occurs in 10% of patients. These toxicities were usually mild and reversible after the end of therapy.

Conclusions

Its modest toxicity profile and the easy schedule of administration make gemcitabine an ideal agent for consideration in the development of chemotherapy regimens. In particular, it would be interesting to evaluate the use of two different nucleoside analogs (fludarabine or pentostatin plus gemcitabine) in modulating the entry route into DNA and their action in terms of direct cytoxicity and apoptosis, respectively. Earlier investigations demonstrated the possibility of potentiating fludarabine with low doses of gemcitabine.[16] In addition, administration of antibody Campath-1H, which has demonstrated activity in T-PLL, in combination with gemcitabine may provide a unique mechanism of cell killing and prove to be an effec-

tive regimen in T-cell malignancies. Given the efficacy of regimens combining cytarabine and cisplatin in lymphoid malignancies, and the experience of combinations of gemcitabine with platinum compounds in solid tumors, combinations of gemcitabine with other compounds should be investigated. Preliminary interesting data have been reported by Emmanouilides *et al.*[17] on a gemcitabine, cisplatin and dexamethasone combination in patients with multiply relapsed Hodgkin's and non-Hodgkin's lymphoma.

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Indolent Lymphomas
Chairmen: E. Morra, P. Rossi Ferrini

Rituximab in indolent lymphomas

PHILIPPE SOLAL-CÉLIGNY
Centre J.Bernard, Le Mans, France

New Drugs in Hematologic Malignancies
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Correspondence: Philippe Solal-Céligny, Centre J.Bernard, Le Mans, France.

Rituximab is an engineered chimeric human-mouse monoclonal antibody (MoAb) consisting of human IgG1 Fc and constant kappa regions with murine variable, antigen-binding regions specific for the CD20 antigen. After treatment with murine MoAbs, human antimurine antibodies (HAMA) may appear. They are directed against one or several antigens of the Fc regions. They neutralize the action of the antibody. After a chimeric mouse-human antibody, the development of HAMA is rare (< 3%). In some rare cases, antibodies directed against the murine-human junction i.e human antichimeric antibodies (HACA) may be found after rituximab treatment. Their effects are not known.[1]

CD20 is a B-cell specific antigen and is first expressed at the pre-B-cell stage. Almost all mature B cells express the CD20 antigen. The level of expression is heteregenous: activated B-cells and germinal center B-cells have a high level of expression. With differentiation towards plasma cells, CD20 antigen expression decreases and plasma cells do not express CD20. The CD20 antigen is a 35 to 37 kDa non-glycosylated protein. It is deeply anchored in the B-cell membrane, with amino and carboxy termini into the cytoplasm. Less than 10% of the protein is expressed on the cell surface. Because of its structure, the CD20 antigen is probably a calcium channel. However, the function of CD20 antigen is largely unknown. It is thought to be involved in B-cell activation, the regulation of B-cell growth, and transmembrane calcium flux.[3]

The mechanisms of action of rituximab are summarized in Table 1. The respective role of each of these mechanisms is still debated. After a single infusion of rituximab, peripheral B-lymphocytes counts are decreased to 0 in 3 days. After four weekly infusions, peripheral B-lymphocytes counts recover in 9 to 12 months. Rituximab also depletes malignant and normal B-lymphocytes from lymph nodes, spleen, liver, bone marrow and other organs. As a single therapy, rituximab is given as 4 weekly intravenous infusions at a dose of 375 mg/m^2. After each infusion, the mean maximum serum concentration, the area under the concentration versus time curve (AVC) increases while the clearance markedly decreases. Serum concentrations are highly variable from patient to patient and are inversely related to peripheral B-cell count and tumor bulk. Serum rituximab concentrations correlate positively with clinical response.[5,6]

Although approximately 80% of patients experience treatment-related adverse events (AE) with rituximab, approximately 90% of these events are mild to moderate in severity. Most AE occur during the first hours of the first infusion and include flu-like symptoms. In rare cases, these symptoms may be severe with hypotension and/or bronchospasm and may even be fatal in exceptional cases. Infusion-related AE are more severe in patients with a high-tumor burden and/or a high number of circulating lymphoma cells.[7] Hematologic toxicity is very rare.

Indications for rituximab have been approved in the treatment of relapsing/progressive follicular lymphomas and clinical experience is by far the greatest in indolent lymphomas.

Rituximab as single agent therapy

The results of the main phase I-2 trials of rituximab in the treatment of relapsed or refractory indolent lymphomas are summarized in Table 2. They show similar results with an overall response rate around 50%, and a complete response rate around 6-10%. The median time to progression of responders is between 10 to 13 months.[8-12] A repeat course of rituximab achieved a clinical response in 41% of 56 patients who had relapsed after a first course of 4 doses of rituximab.[13] From these results, rituximab has been tested as

Table 1. Mechanisms of action of rituximab (from ref. #4).

a.	Activation of complement system
b.	Activation of antibody-dependent cell-mediated cellular cytotoxicity
c.	Direct inhibition of proliferation
d.	Induction of apoptosis
e.	Synergy with chemotherapeutic agents

monotherapy in untreated patients with low-grade NHL. Hainsworth *et al*.[14] treated 41 patients with low-grade NHL (26 follicular, 15 small lymphocytic). After the first course of 4 weekly injections, 21 (50%) had an objective response with 2 complete responses. The response rate was similar in follicular lymphomas (52%) and in small lymphocytic lymphomas (57%). Patients with response or stable disease were retreated every 6 months for a maximum total of 4 cycles. Of the 28 patients who have received multiple courses of rituximab, 72% had an objective response (partial response 54%, complete response 18%).[15] We have conducted a phase II trial of rituximab in 50 patients with a previously untreated, low-tumor burden follicular lymphoma. Patients received 4 weekly doses of rituximab (375 mg/m^2). Overall, 41% of patients reached CR/CRu and 30% reached PR for an overall response rate of 80%.[16] Among 32 patients who could be evaluated for bcl-2-JH rearrangement in the blood, 17 became negative 1 month after treatment.[16] All patients were followed-up for at least 2 years. The median PFS for all patients was 20 months. The median duration of response for patients who were bcl-2-JH polymerase chain reaction (PCR) negative after treatment was longer than that of patients who remained positive (25 vs. 13 months, $p<0.01$).

Results were similar in another trial with an overall response rate of 50% among 20 patients with low-grade NHL.[17]

In conclusion, rituximab, as a single treatment, yields a high response rate in follicular NHL. When compared to results achieved in previously treated patients, the overall and CR rates are higher and the relapse-free survival of responders is longer in previously untreated patients. The toxicity is very low; however, most patients relapse.

Rituximab has also been tested in other indolent lymphomas:

1. small lymphocyte NHL have a low expression of the CD20 antigen. At the usual dose of rituximab, the response rate is low.[9] Higher doses, up to 1500 mg/m^2/week, have been tested.[18] The potential role of rituximab in this histologic subtype is still debated;
2. several trials suggest that rituximab is active in Waldenström's disease. In one retrospective analysis of 30 patients, 27% had a ≥ 50% and 60% a ≥ 25% decline in serum monoclonal IgM levels.[19] Rituximab was also active in patients with polyneuropathy associated with anti-myelin;[20]
3. mantle-cell lymphomas (MCL) have a high expression level of CD20 antigen. Since the prognosis of patients with MCL treated with conventional chemotherapy is very poor, rituximab has been tested first as a single agent. In the largest reported analysis, the response rate was 37% in 40 pretreated patients and 38% in 34 previously untreated patients.[21] The median response duration was around 1 year. Other groups have reported similar results.

Table 2. Results of the main phase 2 trials of rituximab monotherapy in patients with relapsed / refractory low-grade (follicular) NHL.

Author (Ref)	*N° of pts*	*N° of previous treatments median(range)*	*Overall response (%)*	*CR rate (%)*	*Median duration (months)*
Maloney[8]	34	2 (1-6)	50	NP	10.2
Mac Laughlin[9]	156	2 (1-10)	48	6	13.0
Piro*[10]	37	NP	57	14	13.4+
Davis°[11]	31	3 (1-7)	43	3	8.1
Foran[12]	70	3 (1-10)	46	3	11

**Patients were treated with 8 weekly cycles of rituximab at a dose of 375 mg/m^2; °patients had bulky disease with ≥ one lesion ≥ 10 cm.*

Rituximab in combination with other biological response modifiers

There is a rationale for combining interferon-α (IFN) and rituximab. IFN increases (i) expression of CD20 antigens, (ii) the production of and the sensitivity to other cytokines, (iii) the cytotoxic activity of NK cells. In one trial in patients with relapsed or refractory low-grade or follicular NHL, the combination of IFN and rituximab yielded a 45% response rate, very similar to that in patients treated with rituximab alone. However, the estimated median response duration was 22.3 months e.g. around twice the median response duration after rituximab alone.[22] There is also a rationale for combining rituximab with other cytokines such as granulocyte macrophage colony-stimulating factor

(GM-CSF) which may increase the ability of NK cells to mediate antibody-dependent cytotoxicity.

Rituximab in combination with chemotherapy

Although rituximab has very promising effects in follicular and other low-grade NHL, patients still relapse and other approaches are required. Besides, there are several *in vitro* studies which clearly suggest synergistic effects between rituximab and some cytotoxic drugs, especially by lowering the thresholds for induction of apoptosis.[23,24] Several trials of combinations of chemotherapy and rituximab in FL have been reported or are on-going.

A phase II study of CHOP chemotherapy plus rituximab utilized a dosing schedule in which rituximab was administered 1 week before a standard 6-cycle CHOP regimen, during CHOP treatment and after the end of the 6 cycles. A clinical response rate of 95% in 40 patients with indolent NHL (55% CR, 40% PR) was achieved.[25] The median duration of response is > 48 months (unpublished data). Several trials of rituximab in combination with fludarabine and mitoxantrone are ongoing in previously treated patients. The preliminary results are encouraging.[26] An intergroup in France is conducting a trial comparing a combined therapy of a CHOP-like regimen and IFN with or without rituximab. The optimal combination of chemotherapy and rituximab remains unsettled.

Rituximab in autologous stem cell transplantation for indolent lymphoma

High-dose chemoradiotherapy followed by autologous stem cell transplantation (ASCT) is an effective treatment of patients wih progressing/relapsing FL as demonstrated by several retrospective analyses and comparisons to historical controls. In this setting, rituximab can be used:

before stem cell collection, in order to (i) decrease the lymphoma tumor burden; (ii) *in vivo* purge the blood from lymphoma cells and thus decrease the contamination of the graft. Several studies carried out on a limited number of patients have shown that rituximab given before stem cell collection yields a graft depleted from lymphomatous cells as assessed by a PCR analysis for bcl-2-J_H rearrangement.[27] The sensitivity of the technique allows detection of one lymphoma cell out of 10^4 to 10^5 cells. Furthermore, rituximab purging does not affect the yield of $CD34^+$ cells that can be collected nor does it modify time to engraftment and hematopoietic recovery;[28]

or after ASCT, in order to treat the residual disease.

A trial conducted by the European Bone Marrow Transplantation group is presently testing, in a 4-arm randomized trial, the potential role for rituximab in ASCT for relapsing or refractory FL by comparing to a control group, a group treated with rituximab before stem cell collection, a group treated after ASCT, and a group treated before collection and after ASCT.

Similarly, several retrospective analyses strongly suggest that intensive therapy with ASCT may improve the prognosis of MCL patients. Rituximab may be used for *in vivo* purging in this setting.

Rituximab, as a single agent has clearly demonstrated its high efficacy in patients with low-grade NHL. The future of this drug is in combination with other active treatments, especially chemo-immunotherapy and autologous stem cell transplantation.

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Indolent Lymphomas
Chairmen: E. Morra, P. Rossi Ferrini

Epratuzumab (hLL2, anti-CD22) immunotherapy of non-Hodgkin's lymphoma

JOHN P. LEONARD
Center for Lymphoma and Myeloma and Division of Hematology/Oncology, Weill Medical College of Cornell University and New York Presbyterian Hospital, New York, NY, USA

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Correspondence: John P. Leonard, M.D., 525 East 68th Street, New York, NY 10021. Phone: international +1.212.742932.
Fax: international +1.212.7463844. Email: jpleonar@med.cornell.edu

While many patients with non-Hodgkin's lymphoma (NHL) can be cured with chemotherapy and radiotherapy, most patients relapse and eventually die of their disease. Clearly, new therapeutic options are needed. Efforts over many years have concentrated on new cytotoxic drugs and modifications in dosing regimens. Using an immunotherapeutic approach, Nadler and colleagues demonstrated that when appropriate lymphoma-related antigens were targeted with a monoclonal antibody, anti-tumor activity was observed.[1] Miller, Levy and collaborators demonstrated regression of disease after patients were treated with monoclonal antibodies directed against tumor-specific idiotypes.[2] Subsequent studies have employed monoclonal antibodies which target pan B-cell antigens which are commonly expressed in tumors from different patients. The development of rituximab, a chimeric anti-CD20 antibody which combines limited toxicity with significant anti-tumor effects, has been a major breakthrough for this modality of cancer therapy and has resulted in its extensive use in clinical practice.[3-6] While the exact mechanism of action of rituximab in lymphoma patients remains unclear, *in vitro* data suggest that immune mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated lysis may be important,[7,8] while other data support the possibility that induction of apoptosis in B-cells by anti-CD20 antibodies or activation of signaling pathways may result in tumor cell death.[9,10]

While rituximab is clearly a valuable treatment option in many clinical settings, only about 50% of patients with low grade lymphoma will have tumor responses, with a median duration of approximately one year.[6] Findings with single-agent rituximab in aggressive NHL are less encouraging, although combinations of rituximab and cytotoxic agents are currently being tested and appear promising.[11] Despite the benefits of rituximab for lymphoma patients, there is still need for further advances in antibody-based therapy. Exciting strategies currently under evaluation in the clinic include combining unlabeled antibodies with chemotherapies, conjugating radioisotopes to monoclonal antibodies, and utilizing antibodies which target molecules other than CD20. Radioimmunoconjugates combine the anti-tumor activity of naked antibodies with targeted radiation. Antibodies directed against CD20 have been most extensively employed, though antibodies directed against a number of antigens are being studied.[12–16] This approach does not generally eliminate the risk of tumor relapse, or necessarily further delay it, though response rates appear to be improved,[17,18] Additional toxicity, especially of the hematologic system, can be an important issue, and patients with limited bone marrow reserve from prior therapy or the underlying disease may be at particular risk. Combination strategies with chemotherapy are more complicated due to overlapping myelosuppressive effects, and may limit this approach to sequential (rather than concurrent) dosing or require stem cell support.[19] Hence, a number of novel immunotherapeutics are under development which target different antigens on tumor cells. Epratuzumab (hLL2) targets the CD22 molecule, which is widely expressed in B-cell tumors and may play a role in cellular proliferation. This agent offers the potential for alternative strategies (either as a single agent or in combination with other treatments) which may potentially benefit patients with lymphoma.

CD22 as a target for immunotherapy

The CD22 antigen is an adhesion molecule in the immunoglobulin superfamily, is expressed by B-cells, and is involved in their activation and interaction with T-cells.[20-24] Expression is initially observed in the cytoplasm of pro-B and pre-B cells, then on the cell surface as B-cells express IgD.[20] The predominant isoform has seven extracellular domains.[25,26] CD22 is rapidly internalized when antibody-bound, is not shed from the cell surface, and can be re-expressed on the cell membrane after modulation.[27,28] Certain immunotoxins and residualizing isotopes (such as yttrium-90) may make effective conjugates with CD22-directed antibodies due to this feature.[29]

Epratuzumab (hLL2)

Goldenberg *et al.*[32] have led the development of the murine LL2 antibody in a number of preclinical and clinical studies. Immunohistochemistry assays demonstrated that the antibody reacted with 50/51 lymphoma specimens of various histologies, with minimal reactivity with normal tissue except the spleen.[30] The Fab' fragment of LL2 has been radiolabeled and is a sensitive tumor imaging agent under evaluation in staging of lymphoma.[31] Both the Fab' fragment and the full LL2 molecule have been labeled with iodine-131 as a radioimmunotherapeutic, with resultant objective tumor responses in patients with refractory NHL.[32] Subsequently, a humanized IgG1, CDR-grafted version of LL2 (epratuzumab or hLL2) was generated, with comparable pharmacokinetic and dosimetric properties to the murine counterpart.[33] Iodine-131 and yttrium-90 labeled hLL2 have been evaulated in both low-dose and myeloablative (with stem cell support) regimens, with clear demonstration of anti-lymphoma effects and manageable toxicity.[34,35] Tumor regression was observed even with very low doses of radioactivity in the labeled LL2 and hLL2, which suggested that the tumor response may be in part related to the antibody moiety and not solely due to radiation. These findings, as well as the numerous potential anti-lymphoma effects of unlabeled monoclonal antibodies, have led to the development of the naked epratuzumab (hLL2) antibody as an NHL therapy.

Phase I/II study of epratuzumab in non-Hodgkin's lymphoma

At the Center for Lymphoma and Myeloma, Weill Medical College of Cornell University and New York Presbyterian Hospital, we have performed a phase I/II clinical trial of epratuzumab (hLL2) in patients with relapsed or refractory NHL. The goals of this study are to determine the safety and toxicity profile of several protein dose levels, evaluate objective response rates (and relate effects to antibody dose), as well as to observe alterations in peripheral blood B-cell levels. Serum epratuzumab pharmacokinetics are being measured, and patients are assayed for evidence of human anti-human antibody (HAHA) responses. Subjects have a histologic diagnosis of a B-cell malignancy (including NHL, acute lymphocytic leukemia and Waldenström's macroglobulinemia) and must have demonstrated tumor expression of CD22 antigen. Measurable disease is required, and patients must have relapsed from or have been refractory to at least one prior regimen of chemotherapy, with no therapy for four weeks prior to study entry. Initial staging includes history and physical examination, laboratory studies, bone marrow aspirate/biopsy and computed tomography scan of the neck, chest, abdomen and pelvis. Patients have been assigned to receive 4 weekly antibody injections ranging from 120 mg/m^2/dose to 1,000 mg/m^2/dose. Acetaminophen and diphenhydramine premedication is given, and infusions are administered over 30-60 minutes. Patients undergo re-evaluation four weeks after completion of therapy, then every three months until evidence of progression, when retreatment may be given.

Preliminary results in the first patients have demonstrated that essentially all toxicities were grade 1, consisting primarily of infusion reactions such as fevers, rigors and hypotension.

Such side effects were unusual, despite the infusion time of 30-60 minutes. No laboratory changes were observed, except for B-cell depletion in some patients. Antibody levels persist for up to 3-4 months after treatment, and HAHA responses have been rare. Objective tumor responses have been demonstrated in patients with follicular NHL and in diffuse large B-cell NHL. Of the initial group of patients treated at what appears to be the optimal dose level (240 mg/m^2/week or greater), six of 13 patients with follicular NHL achieved a complete or partial response, and several of these responses have extended one to two years. In the first 22 patients with relapsed/refractory diffuse large B-cell lymphoma (median age 60 and 3 prior treatment regimens, most with elevated lactate dehydrogenase), 5 objective responses were observed (23%), with three complete responses and one ongoing over 3 years. Second responses have been observed in several patients who were retreated at the time of relapse.

Conclusions and future directions with epratuzumab

The current trial appears to establish that epratuzumab is a well-tolerated and active NHL therapy when administered on a weekly × 4 basis. It will also identify dose levels and NHL subtypes (primarily follicular and diffuse large B-cell) in which therapeutic activity is observed. Ongoing efforts will likely establish efficacy and safety in more narrowly defined clinical settings with additional patients. Other studies include combinations with chemotherapy and other biological agents (such as with rituximab). Additionally, evaluation continues of the radiolabeled hLL2 (epratuzumab) as lymphoma therapy. The poor ultimate outcome for many patients with NHL following current therapies clearly demonstrates the need for newer approaches. Initial results from the development of epratuzumab are promising and suggest that it may offer potential benefits to patients with B-cell malignancies. Extensive work will be required to evaluate issues of dosing and schedule (as with rituximab) as well as combinations with other agents to optimize ultimate clinical use.

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Indolent Lymphomas
Chairmen: E. Morra, P. Rossi Ferrini

The current status of radioimmunotherapy with iodine-131-tositumomab (Bexxar®) for non-Hodgkin's lymphomas

OLIVER W. PRESS
Fred Hutchinson Cancer Research Center, University of Washington Medical Center, Seattle, WA, USA

New Drugs in Hematologic Malignancies
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Correspondence: Oliver W. Press, M.D., Ph.D. Fred Hutchinson Cancer Research Center, University of Washington Medical Center, Seattle, WA, USA. Phone: international +1.206.6671872.
Fax: international +1.206.6671874. E-mail: press@u.washington.edu

The development of effective monoclonal antibody serotherapy is one of the most important advances in lymphoma therapy in the past decade. The successes of rituximab and other antibodies make it clear that *unmodified* antibodies will remain critical components of lymphoma regimens for the foreseeable future. Despite their unequivocal success, however, there are limitations to the efficacy of unmodified antibodies. Half the patients with relapsed indolent lymphomas and two-thirds of the patients with relapsed aggressive lymphomas do not obtain objective remissions with rituximab and only a small percentage of responding patients achieve complete remissions.[1,2] To enhance the therapeutic potency of antibodies, investigators have conjugated them to cytotoxic radioisotopes to target radiotherapy specifically to tumor sites and improve overall and complete remission rates.

Theoretic advantages of radioimmunoconjugates

Several advantages support the use of radiolabeled antibodies for treatment of lymphomas. First of all, the exquisite radiosensitivity of lymphomas makes them ideal targets for radioimmunotherapy. Second, radioimmunoconjugates kill tumor cells predominantly by radioactive emissions and consequently can be effective even in cases in which unmodified antibodies and immunotoxins are ineffective because of defective host immune function, antigen-negative tumor variants, or tumor penetration problems.[3] The β particles emitted by iodine-131 and yttrium-90 are cytotoxic over many cell diameters, permitting eradication of antigen-negative tumor cells by crossfire from neighboring antibody-coated cells. This feature permits killing antigen-negative mutants and cells deep in tumor nodules that are inaccessible to antibody due to penetration barriers.

Iodine-131 has been the radionuclide employed most often for radioimmunotherapy because it is relatively inexpensive, widely accessible, easily conjugated, can be used for both imaging and therapy, and has demonstrated marked clinical efficacy in treating malignancies such as thyroid cancer, and more recently, B-cell lymphomas. In addition, the emission of γ rays permits radioimmunoscintigraphy with iodine-131-labeled antibodies. However, I-131-conjugates are rapidly degraded after endocytosis into tumor cells, and the resultant small molecular weight I-131-metabolites are rapidly released into the bloodstream.[4] In addition, the γ rays emitted by I-131 may present a potential radiation hazard for family members and health care providers, thereby necessitating hospitalization for radiation isolation if very large doses are employed. The relative advantages of iodine-131, yttrium-90, copper-67 and other radionuclides for radioimmunotherapy are hotly debated by investigators, and no consensus has been reached on the *optimal* radioisotope for lymphoma therapy.

Selection of target antigen

It is now clear that radioimmunoconjugates targeting a large variety of antigens on the surface of B-cell lymphomas can induce clinical complete remissions. Factors which should be considered in selecting a target antigen for radioimmunotherapy include its density on the B-cell surface membrane, the magnitude of antigen shedding into the bloodstream, the rate of internalization into cells after antibody binding, the homogeneity of antigen expression from cell to cell, and the avidity of antibody binding to antigen. Of the pan-B-antigens, CD20 has emerged as the preferred target of most investigators. The CD20 antigen is homogeneously expressed on 90-95% of B-cell lymphomas at a density of 50,000-200,000 sites/tumor cell, is

minimally shed into the circulation and therefore there is little free circulating antigen to block delivery of anti-CD20 mAbs to tumor cells, and CD20 is minimally modulated or internalized following antibody binding.[5,6] In addition, anti-CD20 mAbs are capable of directly killing B-lymphoma cells by both complement-dependent and complement-independent mechanisms.[7] Furthermore, anti-CD20 mAbs induce apoptosis in B-lymphoma cells if cross-linked with Fc-receptor bearing accessory cells.[8,9] Although selection of CD20-negative tumor variants has been reported, this appears to occur very rarely with CD20 antibodies, occurring in less than one in 300 cases.

Clinical trials of non-myeloablative radioimmunotherapy with I-131-tositumomab (Bexxar®)

Mark Kaminski has conducted a series of trials at the University of Michigan using the I-131-tositumomab (anti-B1) antibody (Bexxar®).[10-13] Patients initially receive a pre-infusion of unlabeled tositumomab antibody followed by infusion of tositumomab trace-labeled with 5-10 mCi of I-131. Whole body gamma imaging is performed three times over the week following the trace-labeled infusion to estimate the whole body half-time and calculate the dose required for the therapeutic infusion to deliver the maximally tolerated dose of 75 cGy of whole body irradiation (usually 90-150 mCi). The therapeutic infusion is administered 7-14 days later. Kaminski has recently published his updated single institution experience with 59 patients treated at the University of Michigan.[13] Forty-two patients (71%) responded including 20 (34%) with complete remissions. The median progression-free survival for all responders was 12 months, whereas it was 20.3 months for complete responders. Thirty-five of 42 with low grade or transformed low-grade lymphomas responded (83%) compared with 7 of 17 (41%) with *de novo* intermediate grade NHL. Myelosuppression was the dose-limiting toxicity with 20% of patients experiencing grade IV neutropenia at a median of 43 days after therapy and 20% of patients developing grade IV thrombocytopenia at a median of 35 days after radioimmunotherapy. Non-hematologic toxicities were mild, including low grade fevers, chills, fatigue, nausea, and elevated thyroid stimulating hormone levels in five patients (8%). Five patients later developed myelodysplasia and three patients developed solid tumors, though the relationship of these neoplasms to the radioimmunotherapy is unclear.

Vose has recently published a multi-center phase II trial confirming Kaminski's results.[14] In this trial, 27 of 47 patients responded (57%), including 15 patients (32%) with complete responses. Kaminski has also studied 76 newly diagnosed, previously untreated patients with low-grade lymphomas using this same regimen. Ninety-seven percent of the previously untreated patients achieved objective responses and 63% obtained complete tumor disappearance.[15] Most recently, a multi-center pivotal trial using Kaminski's regimen enrolled 60 patients with refractory follicular or transformed lymphomas and observed remissions in 39 patients (65%) including 10 (17%) complete remissions.[16] These trials demonstrate that the majority of patients with indolent or transformed lymphomas will respond to I-131-tositumomab, regardless of whether the patients are newly diagnosed, relapsed, or refractory. However, the overall and complete response rates are highest in previously untreated patients, intermediate in patients with relapsed but chemosensitive disease, and lowest in patients with chemotherapy-refractory lymphoma. Therefore, the entry criteria for protocols must be carefully scrutinized when one attempts to compare the response rates observed in different clinical trials.

Radioimmunotherapy with stem cell transplantation using I-131-tositumomab (Bexxar®)

Our group in Seattle has conducted a series of investigations over the past decade investigating the feasibility of replacing external beam total body irradiation (TBI) with targeted anti-CD20 radioimmunotherapy for stem cell transplantation of patients with relapsed lymphomas.[17-19] Conventional TBI has been an important component of many bone marrow and stem cell transplant regimens for leukemias and lymphomas for many years. However, TBI delivers as much radiation to normal organs as it does to tumor cells. Therefore, in theory at least, it should be preferable to target the radiotherapy selectively to tumor cells using radiolabeled antibodies since more radiation should be delivered to tumor sites and less to normal organs. In initial phase I/II studies, Press administered high doses of I-131-labeled tositumomab antibody to 29 patients with multiply relapsed B-cell lymphomas. Biodistribution studies indicated that the tositumomab (anti-CD20) antibody yielded superior targeting compared with other tested antibodies including MB-1 (anti-CD37), and anti-idiotypic antibodies.[20] A protein dose of 1.7-2.5 mg/kg of tositumomab achieved optimal biodistribution. The maximally tolerated radiation dose of I-131-tositumomab delivered 27 Gy to normal organs with cardiopulmonary toxicity being dose-limiting.

Twenty-eight of the 29 patients required autologous bone marrow or peripheral blood stem cell transplantation to reconstitute hematopoietic function. Twenty-five of 29 patients responded to therapy with objective remissions (86%) and 23 had complete responses (79%). Eleven of the 29 patients (39%) have remained alive and free of any recurrences for 5-10 years without any further therapy.

Although the high complete response rates and prolonged remission durations were very encouraging, more than half the patients eventually relapsed with single agent radioimmunotherapy, even when given at myeloablative doses with stem cell transplantation. To increase the percentage of patients with durable complete remissions, we subsequently conducted a phase I/II study combining I-131-tositumomab therapy with high dose etoposide, cyclophosphamide and HSCT.[19] Fifty-two patients were treated on this protocol which defined the maximally tolerated doses of this combination to be 25 Gy of I-131-tositumomab (1.7 mg/kg), 60 mg/kg etoposide, and 100 mg/kg of cyclophosphamide. Four patients died of opportunistic infections. At the current time the overall survival rate is 85% and the progression-free survival rate is 73% after a median follow-up of two years. These figures were statistically superior in a multivariable analysis to the overall and progression-free survivals (50% [p=0.01] and 38% [p=0.006], respectively) of a non-randomized control group of patients treated at our institution with the same doses of etoposide and cyclophosphamide, but who received TBI rather than I-131-B1.

Summary

Iodine-131-tositumomab (Bexxar®) produces high overall and complete remission rates in patients with B-cell lymphomas, and many patients experience long-term disease-free survival particularly when myeloablative doses of radioimmunotherapy are administered in conjunction with stem cell transplantation. Myelosuppression has been the dose-limiting toxicity, though delayed myelodysplasia and secondary malignancies are also a concern. Further studies will be required to determine the optimal settings for radiolabeled and unlabeled antibodies in the armamentarium of lymphoma therapy.

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Bcr-Abl as a biological target for STI571

JUNIA V. MELO
Dept. of Haematology, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Hammersmith Hospital, London, UK

New Drugs in Hematologic Malignancies
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Correspondence: Junia V. Melo, MD, PhD, Dept. of Hematology, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Hammersmith Hospital, London W12 0NN, UK.

Over 90% of cases of chronic myeloid leukemia (CML) as well as 10-25% of cases of adult acute lymphoblastic leukemia (ALL) are associated with a reciprocal translocation between chromosomes 9 and 22 which produces the Philadelphia (Ph) chromosome, containing the *BCR-ABL* fusion gene. Depending on the breakpoint in *BCR*, one of 3 types of fusion proteins is generated: $P210^{BCR-ABL}$ is characteristic of CML and also occurs in about 1/3 of *BCR-ABL* positive ALL; $P190^{BCR-ABL}$ is found in 2/3 of *BCR-ABL* positive ALL, and $P230^{BCR-ABL}$ is usually, but not always, associated with the very rare Ph-positive chronic neutrophilic leukemia.[1]

All 3 Bcr-Abl fusion proteins exhibit deregulated tyrosine kinase activity compared with the native Abl protein ($P145^{ABL}$). It is now known that Bcr-Abl interferes with a variety of intracellular signaling pathways in the CML cell, by mechanisms represented mainly by protein-protein interactions. Several domains of the chimeric protein have been found by mutational analysis to regulate cellular transformation; the most essential is the SH1 or tyrosine kinase domain in the Abl moiety. Three major mechanisms have been implicated in the malignant phenotype induced by *BCR-ABL*, namely constitutively active mitogenic signaling, altered adhesion to the stroma and extracellular matrix, and reduced apoptosis. The proliferative signal seems to be exerted through various pathways, including the RAS-MAP kinase, JAK-STAT, and the PI3 kinase pathways.[2]

Because CML was the first neoplastic process to be associated with a consistent acquired genetic abnormality, it is by now the best studied molecular model of leukemia. This knowledge, together with the progressive availability of sophisticated biochemical and biophysical technology, offers a unique opportunity to develop rational molecularly targeted therapies for this leukemia. Since the main transforming property of the Bcr-Abl protein is exerted through its constitutive tyrosine kinase activity, direct inhibition of such activity seems to be the most logical means of *silencing* the oncoprotein. The most straightforward molecular approach is thus to use a chemical compound that competes with ATP for its binding in the kinase domain of Bcr-Abl. This is the basis for the design of STI571 or Glivec, a 2-phenylaminopyrimidine that specifically inhibits the Abl tyrosine kinase, as well as the PDGF and the Kit receptor tyrosine kinases, at micromolar concentrations.[3] *Switching off* the Bcr-Abl kinase activity by STI571 results in transcriptional modulation of various genes involved in the control of the cell cycle, cell adhesion and cytoskeletal organization, leading the Ph-positive cell to an apoptotic death.[4] As shown by us and others, the inhibitor selectively suppresses the growth of CML primary cells and cell lines both *in vitro*[5] and in mice.[5-7] As an anti-neoplastic drug, STI571 is now being tested in an oral formulation in several international clinical trials for the treatment of CML and other Ph-positive leukemias (*as presented by others in this Symposium*).

Like for every other anti-cancer drug, the development of resistance is a possible and important concern. Fortunately, this issue began to be studied in the research laboratories even before clinical resistance was identified in some patients. Thus, several mechanisms of resistance to STI571 have by now been detected, including amplification of the *BCR-ABL* gene, overexpression of the P-glycoprotein encoded by the multi-drug resistance (*MDR1*) gene and, more recently, specific point-mutations in the kinase domain of Bcr-Abl which prevent binding of STI571.[8-11] Understanding how resistant clones can be selected *in vivo* will enable us to design alternative therapeutic regimes in

which STI571 is combined with selective cytotoxic drugs or other signal transduction inhibitors.

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Signal Transduction Inhibitors: where are we going?
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STI571 (GLIVEC®) in the treatment of chronic myeloid leukemia

GIANANTONIO ROSTI, SIMONA BASSI, ELENA TRABACCHI
Institute of Hematology and Medical Oncology "L. e A. Seràgnoli", University of Bologna, Italy

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Correspondence: Dr. Gianantonio Rosti, Institute of Hematology and Medical Oncology "L. e A. Seràgnoli", University of Bologna, Italy. E-mail: grosti@alma.unibo.it

Chronic myelogenous leukemia (CML) is a disease in which the acquisition of the Philadelphia chromosome (Ph) is a universal change present in all leukemic cells and in over 90-95% of the patients. The analysis of the junction point of the fusion chromosome between chromosomes 9 and 22 has shown that a chimeric protein (p210 BCR/ABL) is generated by the chromosomal translocation.

The normal p145, c-ABL-generated protein induces cell cycle arrest and apoptosis when overexpressed by cells. On the contrary, the leukemic counterpart, the BCR/ABL-generated p210 protein, when overexpressed induces rescue from apoptosis and continuous cell cycling. CML hematopoiesis proliferates partially independently from the normal growth-control signaling pathways and, due to its genetic instability, progresses to the fatal terminal accelerated/blastic phase.

It has been shown in animal models and leukemic cell lines that the altered expression and function of the p210 protein is important in maintaining the leukemic phenotype: for example, the incubation of p210 positive cells with antisense oligonucleotides the BCR/ABL mRNA, inhibits or blocks anchorage-independent cell growth. Moreover, the transfection of BCR/ABL retroviral vectors into mouse bone marrow has generated a CML-like disease.

In summary, *in vitro* studies and animal models have established that BCR/ABL alone is enough to induce CML, and mutational analysis has established that the tyrosine-kinase activity of the protein is required for its oncogenic activity. CML is, among hematologic malignancies and solid tumours, the ideal disease for a target-directed treatment: the molecular event which leads to the disease is constant, repetitive and pivotal in maintaining the disease and leading it into final progression. Consequently, an inhibitor of p210 should be an effective and selective treatment for CML.

STI571 (GLIVEC)

By the end of the 1980s, synthetic competitive inhibitors of ATP binding with some degree of specificity among tyrosine kinase had been developed. Novartis scientists developed a lead tyrosine kinase inhibitor through the time-consuming approach of random screening (in brief, testing very large compound libraries searching for inhibition of protein kinase). The most potent compound sorted out by Novartis, inhibited both v-abl and the PDGFR kinase.

Mechanism of action of GLIVEC

p210 is a constitutively active kinase that functions by binding ATP and transferring phosphate from ATP to tyrosine residues on various substrates. The activity causes excess proliferation on myeloid cells: STI571 functions by blocking the binding of ATP to the BCR/ABL tyrosine kinase, thus inhibiting the activity of the kinase. In the absence of tyrosine kinase activity, substrates required for BCR/ABL function cannot be phosphorylated.

Preclinical studies

STI571 has been tested in a number of preclinical models, including *in vitro* assays of enzyme inhibition (Table 1), cellular assays of proliferation and *in vivo* assays of leukemia generation.

As shown in Table 1, at submicromolar concentration STI571 inhibits p210 BCR/ABL, p185 BCR/ABL, v-ABL, c-kit and the PDGF receptor, whereas numerous other kinases, shown in the right side of the Table, are not inhibited even at high concentration of STI571.

STI571, at concentrations ranging between 1 and 10 µM, stops or slows the proliferation of BCR/ABL expressing cell lines tested whereas cell lines which

Table 1: cellular selectivity of STI571

Kinase	IC_{50} [μM]	Kinase	IC_{50} [μM]
v-Abl	0.1-0.3	Flt-3	>10
p210$^{bcr-abl}$	0.25	c-Fms and v-Fms	>10
p185$^{bcr-abl}$	0.25	EGF receptor	>100
TEL-Abl	0.35	c-erbB2	>100
PDGF receptor	0.1	Insulin receptor	>100
TEL-PDGF receptor	0.15	IGF-I receptor	>100
c-Kit	0.1	v-Src	>10
		JAK-2	>100

Cell line	IC_{50} [μM]
32D, MO7e (non-transfected)	>10
p210 $^{bcr-abl}$ - or p185 $^{bcr-abl}$ - transfected 32D, MO7e	<1
BaF3 (non-transfected)	>10
BaF3 TEL-Abl	<1
BaF3 TEL-PDGF receptor	<1
BALB/c 3T3 (parental, *ras*-, *src*-, or SV40-transformed)	>10
BALB/c 3T3 v-*sis* (PDGF-autocrine)	0.3

Figure 1. Specific antiproliferative activity of STI571.

do not express p210 are not inhibited (Figure 1).

Minimal inhibition has been observed when normal bone marrow colonies are exposed to STI571: in contrast, the growth of CML colonies is potently inhibited by the action of STI571.

To summarize, the results of preclinical studies indicated that STI571 is an agent capable of selectively inhibiting the growth of p210 positive leukemic cells and, given the acceptable toxicologic profile that emerged from animal studies, would be worth testin in a clinical context.

Phase I studies/chronic phase disease

The first phase I clinical trial began in June 1998: it was a dose-escalation study aimed at defining the maximum tolerated soe (MTD), having as a secondary endpoint clinical efficacy. Eligible patients [chronic phase, refractory or intolerant to recombinant interferon (IFN)] received STI571 once a day, orally. As shown in Table 2, all the 31 patients who received doses of 300 mg/od or higher got a complete hematologic response, generally within 3 weeks. This observation is consistent with phramacokinetic data which showed that at a dose of 300 mg or greater the plasma levels equivalent to the predicted effective *in vitro* levels of 1 μM are achieved A cytogenetic response was seen at doses of 300 mg or higher in 45% of the cases beyond month 5, with 10% having a complete cytogenetic response (Table 3).

TABLE 2: Phase I trial in chronic phase patients who failed an IFN based regimen.

Dose (mg)	Complete Hematologic Responses	Dose (mg)	Complete Hematologic Responses
85	0/4	300	6/6
140	0/3	350	6/6
200	3/9	400	6/6
250	4/7	500	6/6
		600	7/7

Table 3. Cytogenetic responses in phase I trial (chronic phase patients who failed IFN-based regimen.

	Months on STI571		
Dose (mg)	2 mos.	5 mos.	8 mos.
140	0/3	0/2	0/2
200	0/9	1/8	1/8
250	0/7	0/7	1/6
300	2/6	3/6	2/2
350	1/6	1/6	
400	1/6	3/5	
500	2/6	2/3	
600	5/7		

Side effects were minimal and generally WHO grade I or II. As far as myelosuppression was concerned, at doses of 300 mg or greater, grade 2 and 3 reduction of WBC or platelets were recorded in 21% and 8% of patients. Phase I results in chronic phase showed that, even in patients with late chronic phase disease refractory to IFN, the disease still remained dependent (mainly) on p210, giving the very high rate of complete hematologic response and the impressive number of cytogenetic responses.

Phase I studies/advanced phase disease/ Ph$^+$ leukemias

Given the effectiveness of STI571 in chronic phase CML, the protocol was extended to patients with advanced disease (blastic phase, myeloid and lymphoid) and to patients with acute lymphoblastic leukemia beyond 1st complete remission; the dosage of STI571 ranged between 300 and 1,000 mg/od. Results are summarized in Table 4.

Overall, 20/29 evaluable patients got a partial (marrow blasts less than 15%) or complete (marrow blasts less than 5%) response. Among patients in myeloid blast crisis, 11/18 responded to treatment (4/18 CR) whilst 9/11 lymphoid blast crisis patients

TABLE 4: Phase I study in advanced phase. 4a: patients; 4b hematological responses

Enrolled 33	
Median age (range)	48 years (17 - 75)
Sex (M:F)	16:17
Median treatment duration	68 days (range) (1 - 182 days)
CML blast crisis (N)	25
– Myeloid	21
– Lymphoid	4
Ph+ ALL (N)	8

	300 mg	400mg	500mg	600 mg	Total
Mycloid					
PR	1/5	1/2	3/5	2/6	7/18
Marrow CR	1/5	1/2*	1/5*	1/6*	4/18
Total	2/5	2/2	4/5	3/6	11/18
Lymphoid					
PR	0/2	2/4	1/3	0/2	3/11
Marrow CR	1/2	2/4	1/3*	2/2*	6/11
Total	1/2	4/4	2/3	2/2	9/11

* Complete responses

got a response which was partial in 3/11 and complete in 6/11. Unfortunately, all patients with a lymphoid phenotype but one relapsed between day 45 and 117 whereas 20% of the myeloid blast crisis patients continue to maintain a CR, with a follow-up ranging between 101 and 349 days.

p210 plays a less pivotal role among molecular mechanisms active after progression to blastic crisis: however, STI571 has been shown to be an effective single-agent in this setting. Responses are generally short-lived, particularly in the lymphoid phenotype. Mechanisms of resistance to STI571 in blastic phase are currently under investigation. There are already a number of publications dealing with a BCR/ABL gene-amplification-linked mechanism as one of the potentially numerous mechanism of resistance.

Phase II studies

Phase II trials have been initiated worldwide, expanding the number of patients in late chronic phase or with advanced disease treated with STI571. These trials will provide important information on the effectiveness and safety of the drug in larger cohorts of cases. Moreover, a phased III randomized trial between STI571 and IFN+LDAC in newly diagnosed, early chronic phase patients is currently running.

Suggestions

These early clinical experiences with STI571 answer some questions and leave a number of landmark topics on future developments. STI571 is certainly active in chronic phase disease and this suggests the critical role played by p210 during this phase. It is logical to expect that the best results will be obtained in early chronic phase. An unsolved topic is the exact mechanisms which, following the STI571-mediated inhibition of p210, lead to the quantitative reduction of Ph-positive hematopoiesis and to the re-emergence of Ph-negative hematopoiesis. Does this p210 inhibition reduce the proliferation rate or does it exert a pro-apoptotic activity (or both)? If the second mechanism is proved to be prevalent, STI571 might be able to induce a progressive reduction of the Ph-positive clone until its complete disappearance. In advanced disease, STI571 would be employed (before, during, after?) with conventional chemotherapy to implement its therapeutic effect.

STI571 is currently being tested in clinical trials in association with other agents (IFN and LDAC), based on experimental evidences of a synergistic effect of STI571 when studied *in vitro* in combination with these 2 agents.

Other questions deal with the long-term main-

tenance of the responses recorded to date and with safety issues in the long term. Finally, its development in the setting of allogeneic bone marrow transplantation is also under scrutiny: should it be used, and if so, before or after allogeneic bone marrow transplantation for chronic phase disease?

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Signal Transduction inhibitors: where are we going?
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Inhibition of Bcr-Abl tyrosine kinase by pyridopyrimidine derivatives

JIE WU
H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida, USA

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Correspondence: Jie Wu, MD, Moffitt Cancer Center and Research Institute, Tampa, Florida, USA.

Bcr-Abl tyrosine kinase as a therapeutic target in chronic myelogenous leukemia

As a mechanism that remains largely uncharacterized, reciprocal chromosomal translocations could occur in pluripotent hematopoietic stem cells between the Abl gene on chromosome 9 and the Bcr gene on chromosome 22. This t(9;22) translocation gives rise to the Philadelphia chromosome that contains the *Bcr-Abl* fusion gene,[1] which is a cytogenetic characteristic of chronic myelogenous leukemia (CML). Three forms of Bcr-Abl fusion protein have been found with apparent molecular weights of 185-, 210-, or 210-kDa.[2] These three Bcr-Abl proteins have the same c-Abl derived sequence but differ in the length of Bcr-derived sequence. The 210-kDa Bcr-Abl ($p210^{Bcr-Abl}$) is found in almost all cases of CML and in some cases of acute lymphoplastic leukemia (ALL).[3]

Compared with c-Abl, Bcr-Abl has constitutively activated tyrosine kinase activity and abnormal cytoplasmic localization.[4] Hematopoietic stem cells and progenitor cells normally require cytokines for survival and proliferation. Bcr-Abl activates the STAT5, phosphatidylinositide 3-kinase, and Ras signaling pathways, thereby conferring cytokine-independent survival and proliferation of hematopoietic stem/progenitor cells.[5-11] Introduction of $p210^{Bcr-Abl}$ into mouse bone marrow induces CML-like disease in mice, demonstrating that $p210^{Bcr-Abl}$ is a causative factor for CML.[12] Importantly, laboratory experiments have shown that the protein tyrosine kinase activity of Bcr-Abl is essential to its transformation activity.[13-17] Moreover, inhibition of the normal c-Abl tyrosine kinase does not appear to have an adverse effect on normal cells. In fact, a recent clinical trial shows that a Bcr-Abl inhibitor, STI571, is well tolerated in humans.[18] Therefore, Bcr-Abl tyrosine kinase is an excellent, rational therapeutic target for CML. This is best exemplified by the recent approval of STI571 (Glivac) by the US Food and Drug Administration.

STI571 has shown remarkable clinical efficacy in CML, especially for patients in the chronic phase of CML.[18-20] It is certainly the most successful cancer drug so far designed specifically to inhibit a protein tyrosine kinase. However, new problems have been found during clinical trials of STI571. First, it appears that STI571 cannot eradicate $Bcr\text{-}Abl^{+}$ hematopoietic stem cells in most patients.[18] Second, STI571-resistant CML cells have been observed in experiments and in a clinical setting as results of Bcr-Abl gene amplification, Bcr-Abl tyrosine kinase mutation or other cytogenetic changes.[19,21,22] Therefore, development of new Bcr-Abl tyrosine kinase inhibitors or other therapeutic strategies are warranted.

Discovery of PD180970 as a Bcr-Abl tyrosine kinase inhibitor

We have been interested in a multisite docking protein termed Gab2. Gab2 was originally isolated from $p210^{Bcr-Abl}$ transformed Baf3 cells as a SHP2 binding protein.[23] We found that Gab2 was constitutively tyrosine phosphorylated in human K562 CML cells, which express $p210^{Bcr-Abl}$. We had previously observed that Gab1, a multisite docking protein similar to Gab2, was constitutively tyrosine-phosphorylated in v-Src transformed cells. It was reported that the Src family of protein tyrosine kinases, Lyn and Hck, were activated in Bcr-Abl transformed cells.[24] Therefore, we asked whether a Src family kinase might contribute to Gab2 tyrosine phosphorylation in K562. We treated K562 cells with a classical Src tyrosine kinase inhibitor, PP2, and a new Src tyrosine kinase inhibitor, PD180970.[25] PD180970 {6-(2,6 dichlorophenyl)-2-(4-fluoro-3-methyl-phenylamino)-8-methyl-8H-pyrido[2,3-d]pyrimidin-7-one} is a 2-

phenylaminopyrido[2,3-d]pyrimidine class of protein tyrosine kinase inhibitor (Figure 1). We found that PP2 had little effect on Gab2 tyrosine phosphorylation. In contrast, PD180970 almost completely eliminated Gab2 tyrosine phosphorylation in K562 cells. The decrease in Gab2 tyrosine phosphorylation in K562 cells correlated with loss of Gab2-SHP2 interaction. These results prompted us to assess whether PD180970 has Bcr-Abl tyrosine kinase inhibitor activity.

To determine whether PD180970 inhibits $p210^{Bcr\text{-}Abl}$, we treated K562 cells with different concentrations of PD180970 for 12 h. Tyrosine phosphorylation of $p210^{Bcr\text{-}Abl}$, Gab2, and CrkL was then analyzed by immunoblotting following immunoprecipitation of these proteins. The results show that PD180970 inhibited tyrosine phosphorylation of Bcr-Abl, Gab2 and CrkL in K562 cells in a concentration-dependent manner. The IC_{50} obtained from the average of several experiments were 170 nM for Bcr-Abl and about 80 nM for Gab2 and CrkL.

To test whether PD180970 can directly affect $p210^{Bcr\text{-}Abl}$ tyrosine kinase activity, we immunoprecipitated $p210^{Bcr\text{-}Abl}$ from K562 cells and performed in vitro autophosphorylation of $p210^{Bcr\text{-}Abl}$ in the presence or absence of PD180970. We found that PD180970 potently inhibited autophosphorylation of $p210^{Bcr\text{-}Abl}$ *in vitro* with an IC_{50} of about 5 nM. To rule out the possibility that small amounts of other protein tyrosine kinases might be associated with $p210^{Bcr\text{-}Abl}$ in the immunoprecipitates and that PD180970 may be inhibiting these other kinases, we performed the *in vitro* inhibition assay using a recombinant Abl protein purified from *E. coli*. The recombinant Abl kinase activity was assayed by phosphorylation of a peptide substrate. PD180970 was found to inhibit the recombinant Abl kinase with an IC_{50} of about 2 nM. Because *E. coli* does not have endogenous protein tyrosine kinase, inhibition of the recombinant Abl tyrosine kinase provides conclusive evidence that PD180970 is an Abl tyrosine kinase inhibitor.

K562 cells require Bcr-Abl tyrosine kinase activity for survival and proliferation and have been used as a model to study Bcr-Abl inhibitors.[13,14] On the other hand, cell survival and proliferation of the Bcr-Abl- human HL60 promyelocytic cells is independent of Bcr-Abl. These two cell lines were used to evaluate the cellular effects of PD180970. Incubation of K562 cells with PD180970 resulted in cell death in a concentration-dependent manner. PD180970 had no apparent effect on HL60 cells, indicating that the cell killing effect of PD180970 on K562 was not due to non-specific cytotoxicity.

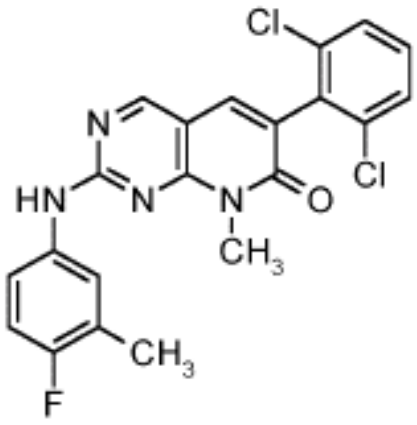

Figure 1. Structure of PD180970.

Cytospins of K562 cells followed by nuclear staining with DAPI showed that PD180970-treated K562 cells had condensed and fragmented nuclei, suggesting that PD180970 caused apoptosis of K562 cells. Analysis of poly(ADP-ribose) polymerase (PARP) indicated that the 116-kDa PARP was cleaved into apoptotic specific 85-kDa and 25 kDa fragments in PD180970-treated K562 cells. Furthermore, annexin V-propidium iodide binding assay confirmed that PD180970 induced apoptosis of K562. Together, our results demonstrate that PD180970 is a potent Bcr-Abl tyrosine kinase inhibitor that can induce apoptosis of Bcr-Abl-dependent cells.[26]

Bcr-Abl tyrosine kinase inhibitor activity of other 2-phenylaminopyrido[2,3-d]pyrimidine derivatives

After our initial finding that PD180970 is a potent Bcr-Abl tyrosine kinase inhibitor, we have tested four other 2-aminopyrido[2,3-d]pyrimidine derivatives, PD159373, PD164199, PD173952, and PD173958, for Bcr-Abl tyrosine kinase inhibitor activity. Three of these compounds, PD164199, PD173952, and PD173958 are 2-phenylaminopyrido[2,3-d]pyrimidine derivatives.[25,27] *In vitro* inhibition studies showed that PD164199, PD173952, and PD173958 inhibited Abl tyrosine kinase and Lyn tyrosine kinase with IC_{50} similar to that of PD180970. In addition to Abl and the Src family kinase, Lyn, these 2-phenylaminopyrido[2,3-d]-pyrimidine derivatives also inhibited Csk. Csk is a tyrosine kinase that phosphorylates Src at the C-terminal negative regulatory site and inhibits the Src tyrosine kinase. PD158373 is not a good

inhibitor of Abl, Lyn and Csk. None of the compounds tested inhibited human insulin receptor tyrosine kinase in our *in vitro* assays.

Consistent with the data from our *in vitro* inhibition study, incubation of K562 cells with PD164199, PD173952, and PD173958 inhibited Bcr-Abl and CrkL tyrosine phosphorylation in K562 in a manner similar to PD180970. In contrast, PD159373 did not have apparent effect on Bcr-Abl and CrkL tyrosine phosphorylation in K562 at all concentrations (up to 1 μM) that we have examined.

To determine whether PD159373, PD164199, PD173952, and PD173958 induce apoptosis of Bcr-Abl-dependent cells, we treated K562 cells and MEG-01 cells, which express $p185^{Bcr\text{-}Abl}$, with these compounds and determined cell viability by trypan blue exclusion assay and apoptosis by PARP cleavage assay. PD180970 was used as a positive control in these experiments. The results show that PD164199, PD173952, and PD173958, like PD180970, induced apoptosis of K562 and MEG-01 cells, whereas PD159373 had no effect.

Together, these results demonstrate that PD164199, PD173952, and PD173958, like PD180970, are Bcr-Abl tyrosine kinase inhibitors that can induce apoptosis of Bcr-Abl tyrosine kinase-dependent CML cells. These four compounds are much more potent than STI571 in inhibiting Bcr-Abl tyrosine kinase *in vitro* and in inducing apoptosis of Bcr-Abl-dependent cells. It remains to be determined whether one or more of these compounds can inhibit the STI571-resistant Bcr-Abl mutant.[21] It also remains to be seen which of these four 2-phenylaminopyrido[2,3-d]pyrimidine classes of Bcr-Abl inhibitors has better pharmacokinetic properties *in vivo*.

Interleukin-3 (IL-3) protects bcr-abl transformed hematopoietic cells against apoptosis induced by bcr-abl tyrosine kinase inhibitors

Although nearly all patients with CML in the chronic phase had complete hematologic response when they were given a sufficiently high dose of STI571, the percentage of patients with complete cytogenetic remission was low.[18] As a consequence, CML patients predictably need to take STI571 for life in order to prevent or delay relapse.

This clinical observation implies that many patients have one or more sub-populations of Bcr-Abl^+ hematopioetic cells that are resistant to the Bcr-Abl tyrosine kinase inhibitor. A well-known growth and survival factor for hematopoietic progenitor cells is interleukin-3 (IL-3). Expression of IL-3 receptor is repressed when hematopoietic progenitor cells differentiate. Interestingly, it was observed that the primitive ($CD34^+$) leukemic cells isolated from Bcr-Abl^+ CML patients have an autocrine loop for IL-3 production and response.[28]

During our studies with PD180970, we found that PD180970 had no effect on cell viability of murine 32D promyeloid cells. The 32D cells are IL-3-dependent. Therefore, they were grown in medium containing IL-3. This observation suggests that PD180970, or a Bcr-Abl inhibitor, does not affect an IL-3-induced cell survival pathway. Thus, the IL-3 autocrine loop found in these primitive $CD34^+$/Bcr-Abl^+ cells could potentially protect these cells against apoptosis induced by a Bcr-Abl tyrosine kinase inhibitor.

To evaluate this possibility, we tested the effects of PD180970, PD164199, and STI571 on $p210^{Bcr\text{-}Abl}$-transformed 32D cells (32D $p210^{Bcr\text{-}Abl}$) and Baf3 cells (Baf3 $p210^{Bcr\text{-}Abl}$). 32D $p210^{Bcr\text{-}Abl}$ and Baf3 $p210^{Bcr\text{-}Abl}$ cells can grow in medium without IL-3, but remain IL-3 responsive. Our results showed that PD180970, PD164199, and STI571 caused cell death of these cells in the absence of IL-3. However, PD180970, PD164199, and STI571 were ineffective in the presence of IL-3. Analysis of $p210^{Bcr\text{-}Abl}$ tyrosine phosphorylation in these cells showed that $p210^{Bcr\text{-}Abl}$ was inhibited regardless of whether IL-3 was present. The IL-3-induced cell survival pathway was believed to be mediated by Jak kinase. Interestingly, PD180970 has been shown to have no Jak tyrosine kinase inhibitory activity.[29]

These results illustrated that the IL-3 autocrine loop found in the primitive Bcr-Abl^+ hematopoietic stem/progenitor cells could be one of the mechanisms responsible for the low efficacy in eradication of Bcr-Abl^+ cells from CML patients by STI571. If this is eventually proved to be the case in clinical trials, one would predict that simultaneous inhibition of the IL-3-induced cell survival pathway and the Bcr-Abl-induced cell survival pathway will offer a permanent cure for CML.

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Signal Transduction inhibitors: where are we going?
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Farnesylproteintransferase inhibitor, Lonafarnib (SCH 66336): a new anti-cancer agent

S.L. Zaknoen
Department of Medicine, Veterans Affairs Medical Center, Minneapolis, MN, USA

New Drugs in Hematologic Malignancies
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Correspondence: S.L. Zaknoen, Department of Medicine, Veterans Affairs Medical Center, Minneapolis, MN, USA

Preclinical summary

SCH 66336 is a tricyclic farnesyl protein transferase (FPT) inhibitor which potently inhibits Ras farnesylation *in vitro* and in intact cells. SCH 66336 also blocks the transformed growth properties (e.g. anchorage–independent growth) of fibroblasts and human tumor cell lines expressing activated H-Ras, N-Ras or K–Ras proteins. Human tumor lines which lack an activated Ras oncogene are also sensitive to soft agar growth inhibition by SCH 66336. This may be due to the presence of transforming events upstream of Ras which require Ras function to induce cellular transformation. Alternatively, it may be due to a role of other farnesylated proteins in cell growth and transformation. Several candidate farnesylated proteins have been identified, including the mitotic kinesin protein CENP-E which functions in chromosome alignment along the metaphase plate. Sensitive human tumor cell lines include carcinoma lines from a variety of tissues (lung, colon, pancreas, prostate, bladder and breast) as well as melanoma, leukemia and astrocytoma cell lines. SCH 66336 also inhibits the soft agar cloning of primary human tumor specimens.

SCH 66336 slows the growth of human tumor cell lines on plastic. This growth inhibition can be enhanced by combining SCH 66336 with other chemotherapeutic agents. A synergistic interaction has been reported when SCH 66336 is combined with taxanes (paclitaxel or docetaxel) or cisplatin. Synergy has also been observed when SCH 66336 is combined with a monoclonal antibody directed against the Her-2/neu receptor (Herceptin) in breast cancer cell lines which overexpress this receptor. Similarly, a synergistic interaction is seen when SCH 66336 is combined with the BCR-ABL kinase inhibitor, STI-571 (Gleevec) to treat primary human chronic myelogenous leukemia cells, including cells which are resistant to STI-571 alone.

Oral administration of SCH 66336 blocks *in vivo* growth of H–Ras and K–Ras transformed cells as well as tumor formation by a variety of human tumor lines grown as xenografts in nude mice. Antitumor activity has been shown in models of lung, pancreatic, prostate, colon and bladder cancer. In an H–Ras transgenic tumor model, SCH 66336 not only inhibited tumor growth, but induced significant regressions of established mammary tumors. Tumor regressions were also observed in two xenograft models (EJ bladder and LOX melanoma). Tumor growth inhibition was also demonstrated in human glioblastoma multiforme xenograft models. In addition, oral administration of SCH 66336 resulted in long-term disease-free survival in mice injected with BCR-ABL-transformed cells and in transgenic mice harboring a BCR-ABL oncogene. SCH 66336 has demonstrated enhanced efficacy in both xenograft and transgenic models when combined with various cytotoxic chemotherapeutic agents including paclitaxel, gemcitabine, cyclophosphamide, 5-fluorouracil and vincristine.

Biochemical pharmacodynamic markers for inhibition of protein farnesylation have been established in preclinical models. These markers are being investigated in ongoing clinical studies. In summary, SCH 66336 is a novel antitumor agent with the potential to have broad clinical antitumor activity. SCH 66336 differs significantly from current chemotherapy in its lack of general cytotoxicity.

FPT inhibition/biochemical activity

SCH 66336 is a tricyclic compound which blocks farnesylation of H–Ras *in vitro* by purified human farnesyl protein transferase (FPT) with an IC_{50} value of 1.9 nM (Table 1). SCH 66336 also inhibits farnesylation of K–Ras–4B *in vitro* with an IC_{50} value of 5.2 nM, while it does not inhibit geranylger-

Table 1. Biochemical pharmacologic evaluation of SCH 66336.

Assay	*IC_{50}*
FPT (H–Ras)	1.9 nM
FPT (K–Ras)	5.2 nM
FPT (N–Ras)	2.8 nM
GGPT–1	>50.0 μM
Cos (H–Ras) (in cell processing)	10.0 nM
m1	>1.0 μM (7%)
m2	>1.0 μM (16%)
m3	>1.0 μM (25%)
m4	>1.0 μM (15%)
m5	>1.0 μM (17%)
H1	>2.0 μM (1%)

SCH 66336 was tested for inhibitory activity in a variety of in vitro enzymic or receptor binding assays. The concentration which resulted in 50% inhibition (IC_{50}) is shown. The prenyl transferases (FPT and GGPT-1) were purified human recombinant enzyme. Various Ras isoforms were tested as FPT substrates. The Cos assay measures H–Ras processing in whole cells as described in the text. The receptor binding assays are for the recombinant human muscarinic receptor subtypes m1 – m5. H1 is the histamine receptor subtype 1. The number in parentheses indicates the % inhibition at the highest concentration tested (1 or 2 μM).

anyl protein transferase type 1 (GGPT–1) at concentrations up to 50 μM. Kinetic studies of compounds in this series demonstrate that they are competitive with the protein substrate (*Bishop et al., 1995*). The interaction of SCH 66336 with FPT has been crystallographically defined at 2.3 Å resolution (*Strickland et al., 1999)*.

SCH 66336 did not significantly inhibit ligand binding to human muscarinic receptors or the human histamine H1 receptor at concentrations up to 1 μM.

SCH 66336 was tested for inhibitory activity in a variety of *in vitro* enzymic or receptor binding assays. The concentration which resulted in 50% inhibition (IC_{50}) is shown. The prenyl transferases (FPT and GGPT-1) were purified human recombinant enzyme. Various Ras isoforms were tested as FPT substrates. The Cos assay measures H–Ras processing in whole cells as described in the text. The receptor binding assays are for the recombinant human muscarinic receptor subtypes m1 – m5. H1 is the histamine receptor subtype 1. The number in parentheses indicates the percentage of inhibition at the highest concentration tested (1 or 2 μM).

Activity of SCH 66336 in models of chronic myelogenous leukemia

The tyrosine kinase oncoprotein BCR/ABL plays a central role in the pathogenesis of chronic myeloid leukemia (CML). BCR/ABL transforms hematopoietic cells through Ras-dependent, as well as Ras-independent, mechanisms. For these reasons, SCH 66336 was tested against murine models of BCR/ABL-induced leukemia and shown to have significant activity.

SCH 66336 potently inhibits soft agar colony formation by a variety of BCR/ABL-transformed cells, including BaF3-P210 cells, a murine lymphoid cell line that is leukemogenic in mice. SCH 66336 slowed proliferation of BaF3-P210 cells in liquid culture and induced apoptosis in a small percentage of cells. Cell cycle analysis revealed that SCH 66336-treated BaF3-P210 cells exhibit markedly prolonged G2/M blockade. Inhibition of Ras activation and blockade of farnesylation of DNAJ (HDJ-2; see below) was demonstrated in SCH 66336-treated cells (*Peters et al., 2001*). In assays comparing bone marrow of healthy individuals and CML patients, SCH 66336 selectively inhibited hematopoietic colony formation of primary human CML cells.

Mice injected with 1×10^6 BCR/ABL-transformed BaF3-P210 cells develop a rapidly progressive leukemia and die within 4 weeks with massive splenomegaly, elevated white blood cell counts and anemia. Treatment of mice with oral SCH 66336 (40 mg/kg b.i.d.) for 34 days prevented the establishment of leukemia and provided long-term disease-free survival in 13 out of 15 mice. Nearly all mice treated with SCH 66336 remained disease-free for over a year. Pathologic analysis of surviving mice demonstrated no evidence of disease (*Peters et al., 2001*).

A separate study was performed in mice carrying the BCR/ABL transgene (p190). These mice develop spontaneous leukemia when BCR/ABL expression becomes activated in peripheral blood cells. SCH 66336 suppressed the leukemic clone and restored BCR/ABL-negative hematopoiesis in the peripheral blood. SCH 66336 treatment prolonged survival in 80% of mice as long as drug treatment was maintained (*Reichert et al., 2001*).

Recently, a small molecule inhibitor of the kinase activity of the ABL protein kinase, STI-571 (Gleevec), received regulatory approval for the treatment of CML. SCH 66336 co-operates with STI-571 in antagonizing BCR/ABL transformation. Furthermore, murine and human BCR/ABL-transformed cell lines that were selected for resistance to STI-571 remain sensitive to SCH 66336 (*Peters et al., 2000*). The combination of SCH 66336 and STI-571 has also been shown to have synergistic or additive effects on the proliferation of STI-571-

resistant cells (*Nakajima et al., 2001*).

Studies from the laboratory of Dr. Alan List at the University of Arizona, USA also demonstrated activity of SCH 66336 against leukemic cells derived from patients with chronic myelomonocytic leukemia (CMML). CMML cells do not express the BCR/ABL fusion protein, but do have a high incidence of activating Ras mutations. Growth of CMML cells in soft agar was potently inhibited by SCH 66336 (IC_{50} = 5 nM). In contrast, growth of committed progenitor cells (CFU-GEMM, BFU-E) was not inhibited by concentrations of SCH 66336 less than 1 μM.

These data support the clinical evaluation of the activity of SCH 66336 in BCR/ABL-induced leukemia and in CMML.

Combination with taxanes

The combination of SCH 66336 with taxanes, both paclitaxel (Taxol) and docetaxel (Taxotere) was tested for its effect on proliferation of human tumor cell lines *in vitro*. Tumor cells were seeded into 96-well plates and allowed to attach for 3 hrs. Cells were incubated with paclitaxel or vehicle for 4 hrs., washed, then SCH 66336 or vehicle was added and the incubation continued for 7 days. Cell proliferation was measured using a tetrazolium-based vital dye. For each cell line, multiple dose response curves were generated for each drug alone and in combination. Data were analyzed using isobologram analysis. SCH 66336 synergized with paclitaxel in 10 out of 11 tumor cell lines originating from breast, colon, lung, ovary, prostate and pancreas (*Shi et al., 1999*). Synergy was observed independently of p53 mutational status, ras mutational status or tissue of origin. Only the breast line MDA-MB-231 failed to show a synergistic interaction. SCH 66336 also synergized with docetaxel in 4 out of 5 cell lines tested, except the MDA-MB-231 line for which where there was no drug interaction (*Shi et al., 1999*). Similar results have been reported with other farnesyl transferase inhibitors (*Moasser et al., 1998*). Those studies indicated that the synergistic interaction is independent of the sequence of drug exposure.

The combination of SCH 66336 and paclitaxel was also evaluated using the NCI-H460 human lung carcinoma xenograft model. Nude mice were inoculated subcutaneously with 3×10^6 tumor cells on day 0. Mice were treated orally with vehicle or 20 mg/kg SCH 66336 twice a day from days 4-14. Intraperitoneal paclitaxel (5 mg/kg) or vehicle was given once a day on days 4-7. Treatment with oral SCH 66336 alone or intraperitoneal paclitaxel alone caused tumor growth inhibition of 52% and 61%, respectively, at the end of the study. Combination treatment resulted in 86% inhibition of tumor growth and was more effective than either single agent ($p < 0.05$).

This combination was also tested in the *wap-ras* transgenic mouse model. Tumors in the *wap-ras/F* substrain are resistant to paclitaxel alone. Mice with palpable tumors were randomized into four treatment groups: vehicle controls, 20 mg/kg SCH 66336 (orally, b.i.d. for three weeks), 5 mg/kg paclitaxel (intraperitoneally, once daily on days 4-7), and the combination of SCH 66336 and paclitaxel treatment. Paclitaxel alone displayed no antitumor activity under these conditions. Oral treatment with SCH 66336 alone resulted in nearly complete inhibition of tumor growth. More importantly, SCH 66336 was able to sensitize the tumors to paclitaxel therapy. The combination of SCH 66336 and paclitaxel was more effective than SCH 66336 alone ($p \leq 0.06$). Tumors in mice treated with both drugs underwent regression over the first 8 days of treatment. Neither single agent treatment induced tumor regressions in this study.

The synergistic interaction between SCH 66336 and paclitaxel might be partially explained by the observation that both compounds lead to accumulation of human tumor cell lines in the G2/M phase of the cell cycle. Inhibition of farnesyl protein transferase might enhance the mitotic block induced by taxol. These results support the clinical exploration of combination therapy using SCH 66336 and taxanes in cancer patients.

Combination with cisplatin

The combination of SCH 66336 and cisplatin was explored *in vitro* using several human tumor cell lines (*Adjei et al., 2001*). A synergistic interaction was observed using A549 human non-small cell lung carcinoma cells and T98G human glioblastoma cells. Synergism was achieved at clinically relevant concentrations of both agents. The synergistic interaction was sequence-dependent, with best results seen when SCH 66336 treatment preceeded treatment with cisplatin. Combination treatment resulted in enhanced apoptosis, however SCH 66336 had no effect on platinum-DNA adduct formation. SCH 66336 appears to enhance the downstream response to these lesions. The synergistic interaction was not seen in all cell lines. A less than additive interaction was observed in MCF7 breast, HCT 116 colon and BxPC-3 pancreatic tumor cells. Additional preclinical studies exploring the combination of SCH 66336 with cis-

platin and carboplatin *in vitro* and *in vivo* are in progress.

Clinical program

The clinical program first focused on tumor types which overexpressed mutant ras including; prostate cancer, melanoma, colorectal cancer and bladder cancer. Phase 2 studies in these indications are either completed or still ongoing. A clinical program in advanced leukemia has begun to explore the striking activity seen in the preclinical CML models. In addition, a program to exploit the preclinical synergy observed with the taxanes and cisplatin has begun and includes trials in first line non-small cell lung cancer and squamous cell carcinoma of the head and neck.

Conclusions

SCH 66336 is a novel oral anti-cancer agent with broad spectrum activity in *in vitro* and *in vivo* models. The toxicities of nausea, vomiting and diarrhea are usually mild to moderate and controlled with standard antiemetic and antidiarrheal medication. The safety of combining SCH 66336 with other chemotherapeutic agents is currently under study. A phase II/III program is ongoing in a number of key indications.

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Therapeutic potential of proteasome inhibitors in hematologic malignancies

Q.P. Dou
Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA

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Correspondence: Q. Ping Dou, Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL 33612, USA.
Phone: international +39.1.81.6321437. Fax: international +39.1.39796748. E-mail: douqp@moffitt.usf.edu

Dysregulation of cell death in human tumors. One important feature of human cancers is their decreased rates of cell death (apoptosis). The phenotype associated with this *too-little-death* problem is drug resistance. Too-little-death in cancer cells partially results from increased levels of some death-inhibitory (oncogenic) activities. *bcr-abl* is one of the death inhibitor classes of oncogenes. Overexpression of *bcr-abl* is found in chronic myelogenous leukemia,[1] resulting in resistance of these cells to apoptosis induced by serum deprivation, irradiation, and chemotherapeutic agents.[2,3] Overexpression of another cell death inhibitor, *bcl-2* pro-oncogene, has been found in follicular lymphomas, diffuse large cell lymphoma, chronic lymphocytic leukemia, and breast cancer.[4] Too-little-cell death in cancer might also be caused by decreased levels of some death-inducing activities (tumor suppressors). Consistent with this hypothesis, pro-apoptotic Bax protein is mutated in human colon cancer and some leukemias.[5,6] Reduced expression of Bax alone, or low ratios of Bax to Bcl-2, is associated with poor response rates to radio- or chemotherapy in patients with B-cell chronic lymphocytic leukemia,[7] breast,[8] ovarian,[9] cervical,[10] and pediatric cancers.[11] Mutations in p53, a transactivator of *bax*,[12] were also associated with resistance of human tumors to radio- and chemotherapy.[13] Finally, mutations of the cell death gene CD95 (Fas/Apo-1) were found in adult T-cell leukemia (ATL), childhood T-lineage acute lymphoblastic leukemia (T-ALL), urinary bladder carcinomas and non-small cell lung cancer,[14-17] suggesting a role of *fas* mutation in drug resistance development of human cancers.

Regulation of cell death by the proteasome. The ubiquitin/proteasome system includes two distinct steps: ubiquitination and degradation.[18,19] Ubiquitination is the step after which the target protein is tagged by multiple ubiquitin molecules and, therefore, can be selectively recognized by the proteasome complex from other proteins. Degradation of such multi-ubiquitinated proteins occurs on a large 26S proteasome complex.[18,19] Recent studies have demonstrated that the ubiquitin/proteasome-mediated protein degradation pathway plays an important role in regulating the cell death process. This suggests that degradation of some specific cellular proteins plays an essential role in determining whether a cell proliferates or dies. In this article, I shall summarize our studies on the proliferation-inhibitory, cancer-preventative and anti-tumor activities of some synthetic and natural proteasome inhibitors. I shall also discuss the involved molecular mechanisms, potential proteasome target proteins, and the clinical relevance.

Induction of apoptosis in human cancer cells by proteasome inhibitor treatment

We have reported that a synthetic, novel dipeptidyl proteasome inhibitor, CEP1612 [phthalimide-$(CH_2)_8$CH(cyclopentyl)CO-Arg(NO_2)-Leu-H], at low concentrations rapidly activates caspases and induces apoptosis in HL-60 and Jurkat T-cells.[20] Since these two cell lines do not contain functional p53 gene, the induced apoptosis must be p53-independent. The apoptosis-inducing potencies of dipeptidyl proteasome inhibitors match precisely their order of inhibition of the proteasome chymotrypsin-like activity. CEP1612 has greater apoptosis-inducing potency than etoposide and is able to induce apoptosis in human prostate, breast, tongue and brain tumor cell lines. The proteasome inhibitor-induced apoptosis is associated with accumulation of cdk inhibitors p21 and p27.[20]

Recent experiments have demonstrated that apoptosis can be triggered by release of mitochondrial cytochrome c into cytosol, which in turns induces activation of effector caspase pathway.[21] To

study whether proteasome inhibitor-induced apoptosis is cytochrome c-dependent, we treated human Jurkat-T cells with the tripeptidyl proteasome inhibitor LLnV, followed by measurement of apoptotic death, PARP cleavage and cytochrome c release. The LLnV-induced PARP cleavage started at 4 h. Levels of cytosolic cytochrome c were also increased at 4 h after the addition of LLnV, and further increased afterwards. The kinetics of cytochrome c release is similar to that of PARP cleavage, suggesting that proteasome inhibition-induced apoptosis is cytochrome c-dependent.

We hypothesized that Bax is a direct target protein of the ubiquitin/proteasome pathway and that inhibition of this pathway by a proteasome inhibitor should accumulate Bax protein that would in turn induce cytochrome c release. To test this hypothesis, we measured the levels of Bax protein by Western blotting. Expression of Bax protein was increased after 1 h LLnV treatment, and further increased afterwards. Therefore, increased levels of Bax protein after LLnV treatment occur prior to release of cytochrome c and induction of apoptosis.

We also measured the subcellular localization of Bax by immunohistochemistry. In untreated Jurkat cells Bax protein was primarily located in the cytoplasm. Treatment with lactacystin markedly increased Bax-associated immunofluorescence in the cytoplasm, which was consistent with the results obtained from Western blotting. The increased Bax-associated immunofluorescence remained largely clustered around the nuclei, suggesting accumulation of Bax in mitochondria. When cells were treated with LLM, a weak proteasome inhibitor but a strong calpain inhibitor, none of the above events, including cell death, PARP cleavage, cytochrome c release and Bax accumulation, was observed.[22]

If Bax is a direct target of the ubiquitin-proteasome pathway, inhibition of proteasome activity should result in accumulation of ubiquitinated Bax protein. To investigate this possibility, protein extracts of Jurkat T-cells treated with lactacystin or LLnV were immunoprecipitated with a monoclonal anti-Bax antibody. The prepared Bax immunoprecipitates were then analyzed by a Western blot assay using a polyclonal anti-ubiquitin antibody. Several polypeptide bands including p55 and p47 were detected in the untreated cell lysate. Treatment with lactacystin or LLnV increased levels of both p55 and p47, suggesting that they are polyubiquitinated forms of Bax. To investigate whether proteasome inhibition might also affect Bax mRNA levels, we measured Bax mRNA in lactacystin or LLnV-treated human Jurkat T-cells by reverse transcription polymerase chain reaction (RT-PCR). No up-regulation on Bax mRNA was detected during the process of proteasome inhibition.[22] Taken together, our data indicate that Bax protein degradation is mainly regulated by the ubiquitin/proteasome pathway and that inhibition of this pathway leads to Bax accumulation, resulting in cytochrome c-dependent apoptosis. Previously, another group also reported that Bax protein levels were increased when HeLa or Saos-2 cells were treated with a proteasome inhibitor although its relationship to apoptosis was not investigated.[23]

We have also shown that ester bond-containing tea polyphenols [such as (-)-epigallocatechin-3-gallate or EGCG] potently and specifically inhibit the chymotrypsin-like activity of the proteasome *in vitro* (IC_{50} 86-194 nM) and *in vivo* (1-10 μM) at the concentrations found in the serum of green tea drinkers. Atomic orbital energy analyses and high-performance liquid chromatography suggest that the carbon of the polyphenol ester bond is essential for targeting and thereby inhibiting the proteasome in cancer cells. This inhibition of the proteasome by EGCG in several tumor and transformed cell lines results in accumulation of two natural proteasome substrates, $p27^{Kip1}$ and IkB-α, followed by growth arrest in the G1 phase of the cell cycle.[24] EGCG at higher concentrations induced apoptosis in leukemia and prostate cancer cell lines, associated with accumulation of Bax protein (unpublished result). Our study suggests that the proteasome is a cancer-related molecular target of tea polyphenols and that inhibition of the proteasome activity by ester bond-containing polyphenols may contribute to the previously observed cancer preventative effect of tea.[25-27]

Drug-resistant tumor cells are sensitive to proteasome inhibitors

Although systemic chemotherapy and irradiation are currently the preferred ways of treating human tumors, acquired or *de novo* drug resistance prevents a satisfactory outcome of such treatments. Understanding the molecular mechanisms of drug resistance in human cancers is essential for improving current therapies.

Bcl-2-overexpressing tumor cells. Many human cancer cells overexpressing *bcl-2* oncogene are resistant to radiotherapy and chemotherapy.[28] We found that proteasome inhibitors induced apoptosis in human leukemia Jurkat T-cells overexpressing Bcl-2.[20] Similarly, another group also reported that proteasome inhibitors activated the apoptot-

ic death program in Bcl-2-overexpressing prostate cancer cells, which are also resistant to cytotoxic anti-cancer drugs.[29] We found that after exposure to 30 µM CEP1612 for 3.5 h, ~100% of the Bcl-2-overexpressing Jurkat cells, similar to the vector-transfected cells, exhibited the apoptosis-specific nuclear morphology. In contrast, etoposide failed to induce apoptosis in Bcl-2-expressing cells. Failure of Bcl-2 to inhibit CEP1612-induced apoptosis could be due to the great potency of CEP1612 at the applied concentration. To investigate this possibility, the control vector cells were exposed to 15 µM CEP1612, a treatment that was less potent than 50 µM etoposide. Overexpression of Bcl-2 had much less inhibitory effect on the cells treated with CEP1612 than those treated with etoposide. Although a slightly delayed PARP cleavage was observed in Bcl-2-expressing cells treated with CEP1612 for 5 to 8 h, similar levels of PARP cleavage were detected in both Bcl-2-expressing and the vector cells after 10 h-treatment. By comparison, expression of Bcl-2 almost completely inhibited PARP cleavage induced by a treatment of 50 µM etoposide for up to 11 h.[20] It appears, therefore, that the dipeptidyl proteasome inhibitor CEP1612 initiates the apoptotic death program through a pathway that bypasses Bcl-2 inhibitory function.

It is possible that proteasome inhibition results in accumulation of Bax in mitochondria, which binds to and inhibits Bcl-2 protein, triggering cytochrome c-dependent apoptosis.[21] To investigate this possibility, we measured levels of cytochrome c and Bax in Bcl-2-overexpressing cells after proteasome inhibitor treatment. Consistent with the partial inhibition of PARP cleavage by Bcl-2 observed earlier, release of mitochondrial cytochrome c in Bcl-2-transfected cells was also inhibited slightly 4 hours after treatment. However, very high levels of cytochrome c were found at later time points in both cell lines. Bax protein was accumulated in both Bcl-2-expressing and vector cells. Proteasome inhibition in Bcl-2-expressing cells caused accumulation of Bax protein primarily in mitochondria and increased levels of Bax-associated Bcl-2 protein, supporting the argument that Bax is responsible for inhibiting mitochondrial Bcl-2 and releasing cytochrome c. Bcl-2 protein did not undergo any post-translational modifications, phosphorylation or cleavage, during proteasome inhibition.[22] Therefore, accumulation of Bax protein by proteasome inhibition is likely responsible for the ability of a proteasome inhibitor to overcome Bcl-2-mediated protection from apoptosis.

Bcr-Abl-overexpressing leukemia cells. The chimeric oncoprotein Bcr-abl, a protein tyrosine kinase, mediates cellular transformation and multiple drug resistance.[30] We have found that proteasome inhibition is sufficient to induce cytochrome c-dependent apoptosis in K562 human chronic myelogenous leukemia cells that overexpress Bcr-Abl oncoprotein (ref. #31 and unpublished results). Our data have identified two major mechanisms involved: inhibition of Bcr-Abl function[31] and accumulation of Bax protein.[22]

The level of Bcr-Abl/p210 protein expression was high in exponentially growing K562 cells, as detected by immunoblotting with an anti-c-Abl antibody (derived from a fusion protein corresponding to the carboxyl region of the v-*abl* protein). However, the Bcr-Abl protein expression was slightly decreased after treatment with 50 µM LLnV for 4 h. A significant reduction in Bcr-Abl protein expression was observed after 8 h treatment. In contrast, no or little change in expression of c-Abl/p145 protein was observed in the same samples. When similar lysates were immunoblotted with an anti-Bcr antibody that recognized the amino terminal sequence of Bcr, significant reduction in Bcr-Abl expression was again observed, while the level of Bcr/p160 was only slightly reduced. Importantly, after 8 h of treatment, no apoptotic changes such as PARP cleavage were observed. The level of Bcr-Abl protein expression remained low after 12 h of treatment, and further decreased after 24 h. The process of PARP cleavage started at 12 h and was completed at 24 h. The ability of LLnV to reduce the Bcr-Abl protein expression was about 100-fold greater than that of LLM, which corresponded exactly to their potencies in induction of PARP cleavage. Furthermore, when the specific proteasome inhibitor lactacystin was used, the decrease in the Bcr-Abl levels was again observed prior to apoptosis induction. In contrast, treatment with the specific cysteine protease inhibitor E-64d neither reduced Bcr-Abl protein level nor induced apoptosis.[31] These results demonstrate that inhibition of the proteasome activity leads to a significant reduction of Bcr-Abl protein expression and subsequent induction of apoptosis.

To establish that the decreased Bcr-Abl expression leads to attenuation of Bcr-Abl-mediated protein tyrosine phosphorylation, the lysates, which were prepared from K562 cells treated with 50 µM LLnV for 7 h, were also analyzed by immunoblotting with an anti-phosphotyrosine antibody. Several heavily tyrosine-phosphorylated proteins were observed in control K562 cell lysates, which is typical of Bcr-Abl-expressing cells.[32] A band of ~210

kDa is Bcr-Abl itself since Bcr-Abl undergoes autophosphorylation on tyrosine residues[33] and since this band was not detected in HL-60 cell lysates. After 7 h of LLnV treatment, the levels of tyrosine phosphorylation for nearly all of the major phosphotyrosine-containing proteins, as well as Bcr-Abl itself, were significantly reduced.[31] This demonstrates that LLnV-mediated reduction of Bcr-Abl protein expression in K562 cells leads to inactivation of Bcr-Abl tyrosine kinase function, which is responsible for the decrease in level of Bcr-Abl-mediated protein tyrosine phosphorylation prior to the initiation of apoptotic execution. The molecular mechanisms responsible for proteasome inhibition-induced decrease in Bcr-Abl oncoprotein levels remain unknown. We have found that it is not inhibited by a variety of protease inhibitors, which suggests that the Bcr-Abl level reduction, if due to proteolytic degradation, must be mediated by some unique protease(s). Alternatively, a different mechanism may be responsible. One possibility is that proteasome inhibition might lead to inhibition of *bcr-abl* transcription.

We investigated whether Bax accumulation by proteasome inhibition also contributes to overcoming Bcr-Abl-mediated protection against apoptosis. We found that cytochrome c was released after 12 h of treatment with LLnV, prior to cleavage of PARP at 16 h. The levels of Bax/p21 protein were increased at as early as 4 h and reached peak level at 12 h post-treatment. A polypeptide(s) of 47 kDa was accumulated prior to cytochrome c release, which might be an ubiquitinated form of Bax since a similar polypeptide was detected by antibodies to Bax and ubiquitin in our immunoprecipitation experiment (*unpublished data*). These results were also in agreement with the immunostaining data, which showed a dramatic increase of Bax immunofluorescence signal in mitochondria following proteasome inhibition. Therefore, both inactivation of Bcr-Abl function and accumulation of Bax protein are responsible for the ability of a proteasome inhibitor to overcome Bcr-Abl-mediation protection from apoptosis. Other groups have also reported that lactacystin treatment sensitized chemo- and radio-resistant CLL cells to tumor necrosis factor (TNF)-α-induced apoptosis.[34,35]

Suppression of human tumor growth by proteasome inhibitors in xenograft models

Given that the proteasome is required for proliferation and survival of tumor cells, inhibition of its activity should arrest or retard cancer progression *in vivo* via inhibition of cell cycle progression and/or induction of apoptosis. Indeed, several groups including ours have recently extended the studies of anticancer activity of proteasome inhibitors from *in vitro* human cancer cell lines to *in vivo* human xenograft animal models.[36-38]

We reported[38] that the dipeptidyl proteasome inhibitor, CEP1612, induced apoptosis and inhibited tumor growth of the human lung cancer cell line A-549 in an *in vivo* model. In cultured A-549 cells, CEP1612 treatment resulted in accumulation of two proteasome natural substrates, the cyclin dependent kinase inhibitors $p21^{WAF1}$ and $p27^{KIP1}$, indicating its ability to inhibit proteasome activity in intact cells. Furthermore, CEP1612 induced apoptosis as evident by caspase-3 activation and poly(ADP-ribose) polymerase cleavage. Treatment of A-549 tumor bearing (s.c.) nude mice with CEP1612 (10 mg/kg per day, i.p. for 31 days) resulted in massive induction of apoptosis (TUNEL) and significant 68% tumor growth inhibition ($p<0.05$). Furthermore, immunostaining of tumor specimens also demonstrated *in vivo* accumulation of $p21^{WAF1}$ and $p27^{KIP1}$ following CEP1612 treatment. The results suggest that CEP1612 is a promising candidate for further development as an anticancer drug and demonstrate the feasibility of using proteasome inhibitors as novel antitumor agents.

Conclusions

The involvement of the ubiquitin/proteasome-dependent degradation pathway in up-regulation of cell proliferation, down-regulation of cell death, and development of drug resistance in tumor cells suggests the potential use of proteasome inhibitors as novel anticancer drugs. This hypothesis has been supported by both *in vitro* and *in vivo* experimental results as well as phase I clinical results.[39,40]

Given the fact that the proteasome is involved in several important cellular processes, it will be a great challenge for researchers to design specific, selective and potent proteasome inhibitors. Ideally a proteasome inhibitor to be used for therapeutic purposes should not inhibit other protease activities, interact with other proteins, or affect normal cells at therapeutic doses. It would be important that the proteasome inhibitor selectively accumulates only the pro-apoptotic or anti-proliferative proteins (i.e., Bax, p53, p27), but not the anti-apoptotic or pro-proliferative proteins (i.e., Bcl-2, MDM2, cyclin E). Such a selectivity of the proteasome inhibitor should be associated with increased potency against tumor cells. Equally important, better understanding of the cancer-related proteasome target proteins will accelerate

the drug discovery process of novel proteasome inhibitors. It is to be hoped that more specific, selective and potent proteasome inhibitors will be developed in laboratories and used in cancer therapies in the near future.

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Hitting New Pathways

Chairmen: F. Lo Coco, A. Zaccaria

PS-341: an emerging role in the treatment of multiple myeloma

P. RICHARDSON

Departments of Adult and Pediatric Oncology, Dana-Farber Cancer Institute, Brigham and Women's Hospital and the Children's Hospital, Harvard Medical School, Boston, Mass., USA.

Correspondence: P. Richardson, Departments of Adult and Pediatric Oncology, Dana-Farber Cancer Institute, Brigham and Women's Hospital and the Children's Hospital, Harvard Medical School, Boston, Mass., USA.

PS-341 (also known as LDP-341) is a novel small molecule that is currently being assessed in clinical trials of the treatment of various solid tumors and hematologic malignancies. PS-341 has a novel mechanism of action through inhibition of the so-called *proteasome*. The proteasome is an enzyme found in all cells of the body and plays a key role in protein degradation, cell cycle regulation, and gene expression. Normal cell cycle regulation is mediated by a diverse array of proteins. The effects of such proteins, whether positive (growth stimulating) or negative (growth inhibitory), depend on the presence of these proteins as critical steps in cell cycle regulatory pathways. The proteasome plays a pivotal role in this process by degrading proteins; consequently, proteasome inhibition can lead to cell cycle dysregulation and ultimately so-called *apoptosis* (programmed cell death).

Myeloma cells seem to be more sensitive to the pro-apoptotic effects of proteasome inhibition than are normal cells. Normal cell growth and differentiation mechanisms are highly sophisticated, containing numerous checkpoints at which the cell can repair potentially lethal (or transforming) events to maintain viability. Although transformed cancer cells have acquired genetic mutations that allow for a growth advantage, they also have lost many of the checkpoint mechanisms that most normal cells have. This loss of checkpoint mechanisms can be viewed as a critical vulnerability that can be preferentially exploited. Pre-clinical studies suggest that cancer cells, and in particular myeloma cells, are more sensitive than normal cells to the pro-apoptotic affects of proteasome inhibition, possibly because they have lost key checkpoint mechanisms that in normal cells help repair cell protein dysregulation.

Several pathways to prevent apoptotic cell death in tumor cells exist. One example especially relevant to multiple myeloma is the activation of NF-κ B, a transcription factor. When myeloma cells are activated or sustain DNA damage due to chemotherapy or radiation, experimental evidence shows that NF-κ B activity is promoted. This process is in part controlled by the proteasome which degrades an important protein regulator of NF-κ B, called *I -κB*. When the proteasome is activated and I -κ B is degraded, NF-κ B is released. NF-κ B then translocates to the nucleus, binds to DNA and initiates the transcription of key growth factors, such as interleukin and tumor necrosis factor α; angiogenic factors, such as vascular endothelial growth factor (VEGF), and cell adhesion molecules, such as vascular-cell adhesion molecule (V-CAM). In turn, myeloma cells bind to stromal cells; this normally protects the tumor cells from apoptosis but also results in increased production of growth factors and angiogenic factors. Thus myeloma cells grow and proliferate in response to NF-κ B mediated events. However, in the presence of PS341, the proteasome is inhibited and this prevents the degradation of the NF -κ B/I -κ B complex, which in turn abrogates the effects of NF-κ B on growth, proliferation and gene expression. Inhibition of the proteasome ultimately therefore leads to cell cycle arrest, apoptosis and the myeloma cell dies. In aggregate, these laboratory studies have shown that proteasome inhibition has several pro-apoptotic effects on myeloma cell viability, one of which is prevention of the activation of NF-κ B, a key transcription factor, and this in part may explain the activity of PS341 in multiple myeloma. Furthermore, in pre-clinical studies, PS341 has not only reduced tumor burden when given alone but also has been shown to have at least additive effects when combined with other therapeutic agents, including

dexamethasone.

PS341 has now been assessed in a variety of solid tumors and in hematologic malignancies, including myeloma. In preliminary trials, PS341 dosing has been guided by biological and anti-tumor activity and a pharmacodynamic assay, which has been specifically designed to quantify the percent proteasome inhibition seen in various tissues. Preliminary reports indicate that biologically active doses of PS341 have been associated with both responses and manageable toxicity in myeloma patients as part of phase I studies. Multicenter trials of PS341 as monotherapy and in combination with dexamethasone in patients with multiple myeloma are now underway, with phase II studies of PS-341 in combination with standard chemotherapeutic agents for other cancers also in process. Regimens consisting of doses between 1 mg and 1.3 mg/m^2 given twice a week, at least 72 hours apart, for 2 weeks with one week off, for up to 6 months are being tested as part of the phase II trials in relapsed and relapsed, refractory multiple myeloma. Side effects have included fatigue, some suppression of blood counts including white blood cells and platelets, occasional fever and skin rash, and possibly exacerbation of pre-existing nerve damage. So far, these clinical studies have shown evidence of anti-tumor activity with an acceptable safety profile. Given the heavily pretreated nature of the patients and the responses seen to date, PS341 appears to have considerable promise as a new biologically derived therapy in the treatment of multiple myeloma.

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Hitting New Pathways
Chairmen: F. Lo Coco, A. Zaccaria

Histone deacetylase inhibitors: new therapies for cancers

PAUL A. MARKS
Memorial Sloan-Kettering Cancer Center, and
Columbia University, New York, USA

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Correspondence: Paul A. Marks, M.D. Memorial Sloan-Kettering Cancer Center, New York, NY, USA. E-mail: paula_marks@mskcc.org

Neoplastic transformation does not necessarily destroy the potential that cells have to differentiate and/or undergo apoptosis, under appropriate environmental conditions.[1] It is well established that various chemical agents can induce these processes in transformed cells[2-5] Among the most effective of these are histone deacetylase (HDAC) inhibitors.[4,5]

Histone proteins organize DNA into nucleosomes which are regular repeating structures of chromatin. The acetylation status of histones alters chromatin structure which, in turn, regulates gene expression.[6,7] Two classes of enzymes can affect the acetylation of histones-histone acetyl transferases (HATs) and histone deacetylases HDACs.[6-9] Altered HAT or HDAC activity has been identified in several cancers.[10-13]

A number of HDAC inhibitors have been characterized that inhibit tumor cells growth *in vitro* and *in vivo* at amounts that have little or no toxicity,[4,14-16] and several of these are being tested in clinical trials. These compounds act very selectively on genes whose expression they alter, increasing or decreasing the transcription of fewer than 2% of expressed genes.[17] Recent studies are beginning to elucidate the structural features of protein complexes that regulate gene transcription in normal and transformed cells.[6-13,18-20]

The classes of compounds identified as HDAC inhibitors now includes: 1) short-chain fatty acids (e.g., 4-phenylbutyrate and valproic acid); 2) hydroxamic acids (e.g., SAHA, pyroxamide, (Table 1) TSA, oxamflatin and CHAPs); 3) cyclic tetrapeptides [(trapoxin A, apicidin and depsipeptide (FK-228 a.k.a. FR901228); and 4) benzamides (MS-275). These HDAC inhibitors have been postulated to interact with the catalytic site of HDAC, thereby blocking substrate access to the active catalytic site as evidenced by X-ray crystallographic studies.[21]

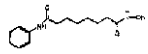
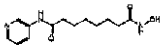
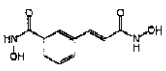
Activity of HDAC inhibitors in vitro

SAHA and related compounds cause cell cycle arrest in G1 and/or G2 phase, apoptosis, and/or differentiation in cultured transformed cells. Growth inhibitory effects have been documented in virtually all transformed cell types, including cell lines arising from both hematologic (leukemias, lymphomas, and myelomas) and epithelial tumors (breast, bladder, ovarian, prostate, lung, and neuronal, etc.). The concentrations of HDAC inhibitors required for cell cycle arrest correlate with the concentration required to cause accumulation of acetylated histones.[15,16] SAHA, and related hydroxamic acid-based hybrid polar compounds are active at micromolar concentrations.

Histones H2A, H2B, H3 and H4 become acetylated following treatment with HDAC inhibitors in both normal and tumor cells.[22] However, the growth-suppressive and apoptotic activities of these agents appear to be confined to transformed cells. Treatment of normal human fibroblasts or melanocytes with the hydroxamic acid-based hybrid polar compounds, azelaic bishydroxamic acid (ABHA) or azelaic-1-hydroxamate-9-anilide (AAHA), causes no growth inhibition at doses that suppress the growth of transformed cell lines.[23,24]

We have been investigating the cellular mechanism of action of SAHA and related compounds. SAHA induces expression of $p21^{WAF1}$, a cyclin dependent kinase inhibitor.[25] $p21^{WAF1}$ inhibits cell cycle progression, acting by blocking cyclin dependent kinase activity and, as a consequence, causing arrest of cells in G1. HDAC inhibitors require the presence of a specific site, the Sp1 site in the promoter of p21 gene. This suggests that the HDAC inhibitor acts directly on the $p21^{WAF1}$ promoter, not on an upstream element of the pathway. SAHA induces accumulation of acetylated histone in the chromatin that is associated with the $p21^{WAF1}$ gene, and this increase correlates with an increase in

Table 1. Histone deacetylase inhibitors.

Name	Structure	Activity** HDAC	Cell culture	Animal tumor models	Clinical trials
Suberoyl-anilide hydroxamic acid (SAHA)		×	×	×	×
Pyroxamide		×	×	×	×
m-Carboxy-cinnamic acid bishydroxamic acid (CBHA)		×	×	×	–

An × indicate that the compound has been shown to have activity in inhibiting partially purified HDAC, growth of transformed cells in culture and, in vivo, tumor growth in animal studies. Clinical trials indicate the agent is in phase I clinical trial. An – indicates no data reported.

transcription of the p21^{WAF1} gene.[25] In our studies, we found that in addition to p21, the HDAC inhibitors induce thioredoxin binding protein-2 (TBP-2) in each of five transformed cell lines examined. TBP-2 binds and inactivates reduced thioredoxin (Trx). Trx is required for activation of a number of proteins involved in DNA synthesis (e.g., ribonucleotide reductase) and gene regulation. Trx is overexpressed in many cancers and we found that TBP-2 expression is low in many cancers.

Based on our studies of the promoter regions of the p21 and TBP-2 genes whose transcription is selectively increased by SAHA, we suggest that there are genes containing specific sites in the promoter region for binding transcription complexes that recruit HDAC, e.g., Sp1 or NF-Y and repress gene transcription. Activation of these silenced genes (e.g., p21^{WAF1} and TBP-2) by inhibition of HDACs could contribute to the growth arrest differentiation and/or apoptosis of transformed cells.

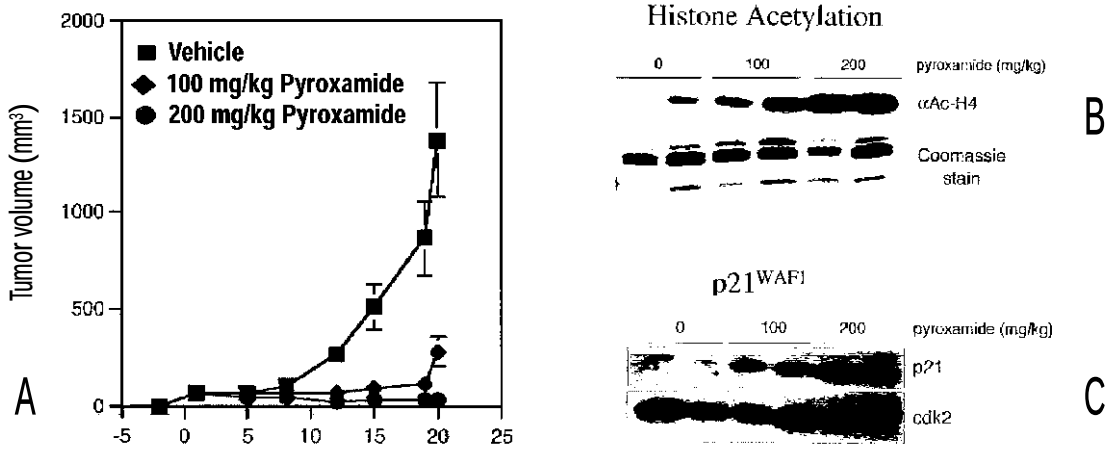


Figure 1. Effect of intraperitoneal pyroxamide, 100 or 200 mg/kg/day, for 21 days on nude mice with transplanted (CRW22) human prostate xenograft. A. Effect of pyroxamide on tumor volume in mice receiving no drug (vehicle) or 100 mg/kg pyroxamide or 200 mg/kg pyroxamide. B. Accumulation of acetylated histone was analyzed by Western blotting of histone extracted from tumors removed from treated animals. The upper panels show the levels of acetylated histone H4 (αAC-H4) detected by polyclonal antibodies, while the lower panel shows a Coomassie blue-stained polyacrylamide gel of the histone samples. C. Expression of p21^{WAF1} was analyzed in whole-cell extracts prepared from tumors from treated animals (vehicle alone, 100 mg/kg/day pyroxamide or 200 mg/kg/day pyroxamide). The upper panel shows p21^{WAF1} expression, and the lower panel shows the levels of cdk2 in the tumors. Cdk2 was used as a loading control because the expression of this protein is not known to be modulated by HDAC inhibitors.[21]

HDAC inhibitors as new cancer drugs

Hydroxamic acid-based hybrid polar HDACs inhibitors, SAHA, CBHA and pyroxamide (Table 1) have been tested extensively in animal studies.[26,27] For example, in nude mice bearing transplanted CWR22 androgen-dependent human prostate tumors treated with SAHA, tumor growth was suppressed as much as 97% compared with growth in untreated tumor-bearing animals. Acetylation of histones H3 and H4 increased in the CWR22 tumor cells within 6 hours after injection of SAHA. Pyroxamide had similar effects on CWR22 tumor growth (Figure 1) as did CBHA on the growth of human neuroblastoma xenografts in nude mice. At doses that markedly inhibited tumor growth, SAHA, pyroxamide or CBHA cause little or no toxicity as evaluated by weight gain, histologic studies and examination of multiple tissues at necropsy.

SAHA and pyroxamide have recently entered clinical trials. Dose-escalating studies have shown that SAHA is safe. Accumulation of acetylated histones can be detected in peripheral mononuclear cells and tumor biopsies following the administration of SAHA. SAHA has shown evidence of efficacy in both patients with solid tumors and with Hodgkin's disease at doses that have no clinical toxicity and studies to define the optimal therapeutic regimen are ongoing. Studies with an oral formulation of SAHA are also underway.

In summary, clinical trials show that HDAC inhibitors are well tolerated, can inhibit HDAC activity in peripheral mononuclear cells and tumors and, more importantly, have clinical activity with objective tumor regression. On a practical, therapeutic level, defining the optimal dose, timing of administration, duration of therapy and other agents to combine with HDAC inhibitors are some of the challenges that must be faced in their continued clinical development.

Acknowledgments

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Index of authors

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