The selective anticancer agents PB-100 and BG-8 are active against human melanoma cells, but do not affect non malignant fibroblasts

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Abstract. When past the stage amenable to surgery, melanoma and its metastases are, as a rule, treated with chemotherapy, which is largely unsuccessful. In this report, experimental evidence is presented demonstrating that, *in vitro*, two selective anticancer agents, PB-100 and BG-8, dose dependently destroy human G-361 melanoma cells, but do not affect human non malignant CCD-974Sk fibroblasts used as controls. Trace metal compounds, present, often in abnormal amounts, in the cancer cell and/or its environment, are known to influence its proliferation. Assays were carried out using highly elevated amounts of ferritin, iron chloride or zinc chloride. Ferritin proved differentially mitogenic for melanoma cells and fibroblasts. Its activity was inhibited by both anticancer agents, which however tended to become less efficacious in its presence. $FeCl_3$ was more moderately, but equally, mitogenic for malignant and normal cells, yet it impaired antiproliferative activity of PB-100 and inhibited that of BG-8. $ZnCl_2$ exhibited a selective antiproliferative activity on the malignant melanoma cells; it did not compete with PB-100 or BG-8. Specific recognition and destruction of malignant cells by the two anticancer agents are discussed.

Introduction

Melanoma has become one of the foremost types of cancer and its incidence is steadily increasing. Though surgical treatment may deal successfully with early stages, patients with larger tumors and especially metastases must resort to chemotherapy, which in melanoma yields only poor and temporary results, in addition to having the major drawback of indifferently damaging both cancer and normal cells and thus leading to severe adverse side effects (1-3). The need for selective anticancer drugs is now widely recognized, and treatment of melanoma remains a specific challenge.

Over the years, we have developed a number of anticancer agents which proved active *in vitro* and *in vivo* on various types of malignant cells, while in no way affecting normal, non malignant cells (4-8). Of these agents, the ones we most thoroughly studied are PB-100 and BG-8.

In the general course of our research on differential cancer and non-cancer cell responses, we also studied the effects on malignant and normal cells of such essential inorganic ions as iron or zinc. Using a human glioblastoma and a normal astrocyte cell lines, we demonstrated some time ago that, *in vitro*, ferritin was more highly mitogenic for the malignant than the normal cells; in contrast, zinc exhibited antiproliferative activity (9).

In assays described in this report, we studied *in vitro* the activity of PB-100 and BG-8 on the human melanoma G-361 cell line, using as a control the human non malignant CCD-974Sk fibroblast line. Then we investigated the effects of ferritin, $FeCl_3$ and $ZnCl_2$ on these two cell lines, and tested PB-100 and BG-8 activities in the presence of these molecules.

Materials and methods

Chemicals. $ZnCl_2$ (grade A) and $FeCl_3$ (crystalline), (Prolabo, France). Ferritin (ICN Biochemicals, Cleveland, OH, USA). Trypsin, RPMI 1640 culture medium, fetal calf serum, glutamine (Gibco, Grand Island, NY, USA). Other chemicals (grade A) (Prolabo, France).

Anticancer agents and DNAs. The plant-derived alkaloids PB-100 and BG-8 were purified in our laboratory; purity was checked using HPLC, UV absorbance spectra and thin layer chromatography. DNAs from 48 h cultures of human melanoma cells and normal fibroblasts were isolated, purified and analyzed according to the method described elsewhere (5). *In vitro* binding of PB-100 and BG-8 to cancer and normal DNAs was measured as previously described (10,28).

Cell lines and culture techniques. Human melanoma cell line G-361 and human normal (i.e., non malignant) fibroblast line CCD-974Sk were obtained from the American Type Tissue Culture Collection, Rockville, MD, USA. After absence of

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; PB-100, flavopereirine; BG-8, alstonine

Key words: melanoma, fibroblasts, PB-100, BG-8, alkaloids, nucleus, nucleolus

mycoplasms or bacteria was checked, cells were grown at 37°C as monolayer confluent cells in RPMI 1640 medium supplemented with 10% calf serum. To avoid cell membrane sensitization, no antibiotics were used. Stock cultures were duplicated weekly after addition of trypsin (+0.05% EDTA) to disperse cells for inoculation. Both lines were grown for 48 h (doubling time: 20-24 h) prior to assays. Each cell type was cultured in several 6-well tissue culture plates (9.8 cm^2 wells) starting from a $4x10^4$ inoculum.

Cell growth inhibition and stimulation. Antiproliferative effect of PB-100 and BG-8, stimulatory activity of ferritin and $FeCl_3$, and inhibitory effect of $ZnCl_2$ were determined following addition of increasing concentrations of these substances to culture medium already containing cell inoculum. Dead cells were found in suspension, while remaining viable cells were trypsinized and then counted with a Coulter counter.

Short treatment of melanoma cells and fibroblasts with PB-100 or BG-8. Cells were grown for 48 h and 1 mg/ml PB-100 (sterilized distilled water solution) was then added. After a 10 min incubation, aliquots of treated melanoma cells and untreated fibroblast controls were removed for further culture after dilution with fresh medium, and were examined with an inverted microscope under visible light or UV (270-380 nm).

Results

Assays of PB-100 and BG-8 on G351 melanoma cells and CCD-974Sk fibroblasts. Effects of PB-100 and BG-8 on melanoma cell and fibroblast multiplication. Activities of PB-100 and BG-8 are dose-dependent. Following a 48 h incubation in the presence of PB-100 (100 µg/ml), 95-98% melanoma cells were killed by the anticancer agent; under identical conditions, up to 80-90% melanoma cells, on the average, were killed by BG-8 (100 µg/ml) (Fig. 1). In addition, malignant cells which escaped destruction have apparently lost the ability to multiply. Microscopic examination of dead cells that have detached from the culture wells showed they had enlarged compared to untreated controls and their membrane had collapsed in places. There were signs of pycnosis in the nucleus, and cytoplasm had become granular (Fig. 2A and B).

By contrast, under identical experimental conditions, non malignant fibroblasts continued to multiply normally in the presence of either of the two anticancer agents (100 µg/ml), and remained unaltered (Fig. 2A and Fig. 3A). These data confirm that PB-100 and BG-8 are selectively toxic for the malignant melanoma cells, but do not affect the normal fibroblasts. Such results confirm and extend those we previously obtained using malignant and normal cells of different origins (6,11,12). PB-100 and BG-8 do not compete with each other (data not shown).

Microscopic evidence for anticancer agent cellular localization. After a 10 min exposure to PB-100 or BG-8, microscopic examination under visible light shows that, in melanoma cells, the anticancer agents, easily detected by their yellow color, very rapidly accumulate in the nucleus.

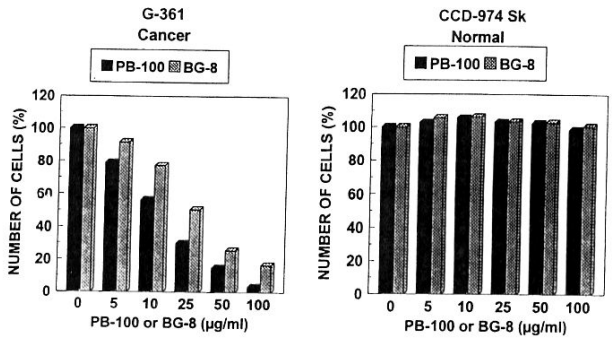

Figure 1. Effect of PB-100 and BG-8 on the multiplication of human melanoma cells and normal human fibroblasts. Both cell lines were grown in triplicate for 48 h at 37°C in the absence and presence of indicated concentrations of PB-100 and BG-8. Data are an average of three separate experiments. G-361, SD ± 4.2; CCD-974Sk, SD ± 3.85 (PB-100); G-361, SD ± 5.3; CCD-974Sk, SD ± 4.69 (BG-8).

PB-100 particularly concentrates in the nucleoli (Fig. 2B), while BG-8 evenly stains the whole nucleus and does not apparently penetrate nucleoli (Fig. 3B). When tested on fibroblasts under the same conditions, neither PB-100 nor BG-8 can enter these normal cells; these are simply surrounded by the anticancer agents, which remain outside (Fig. 2A and Fig. 3A), and the cell membrane is not altered.

Both PB-100 and BG-8 exhibit a blue fluorescence under UV illumination (270-380 nm). Observations confirm that, in melanoma cells, PB-100 rapidly colors the nucleus and especially the brightly staining nucleoli (Fig. 2C); it is apparently absent from the cytoplasm. Using BG-8 (Fig. 3C), the whole nucleus becomes evenly blue, while the cytoplasm is slightly stained as well. Fibroblast contours are underlined, as the agents cannot enter these cells, and neither their nucleus nor their cytoplasm are stained.

Selective binding of PB-100 and BG-8 to melanoma cell DNA. Measurement of 260 nm UV absorbance of purified DNAs from melanoma cells and fibroblasts show that this absorbance is higher (hyperchromicity) for the malignant than for the non malignant DNA (10). This confirms results of UV absorbance measurement we systemically carried out for almost 20 years on cancer and non-cancer DNAs (10). Hyperchromicity is a sign that there are multiple broken H-bonds in the cancer DNA molecule (see Discussion). In the presence of increasing concentrations of PB-100 or BG-8, melanoma DNA hyperchromicity gradually disappears, indicating that, as a result of anticancer agent binding, strands separated by H-bond breakage are brought back together; this process goes on until cancer DNA secondary structure collapses. In contrast, fibroblast DNA UV absorbance does not change. The two anticancer agents thus selectively bind to the malignant melanoma DNA, corroborating the above-described microscopic observations (Fig. 4).

Assays of PB-100 on G351 melanoma cells and CCD-974Sk fibroblasts in the presence of ferritin, iron chloride and zinc chloride. Effects of ferritin and $FeCl_3$ on melanoma cell and fibroblast multiplication. Following a 48 h incubation in the

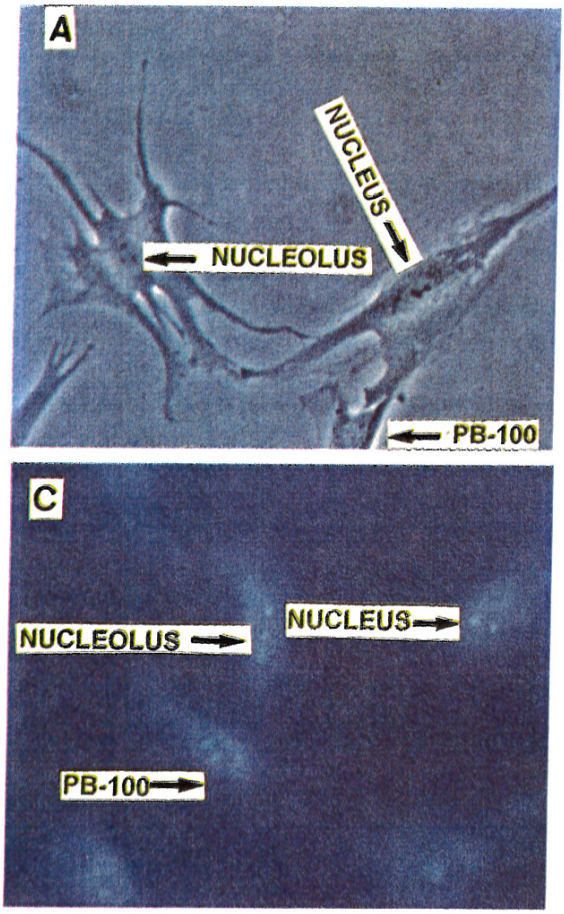

Figure 2. Visible light and UV microscope observations of PB-100 treated cells. A, Normal fibroblast controls treated with 1 mg/ml PB-100 for 10 min at 37°C during growth phase. The anticancer agent remained outside these cells, as shown by the halo that surrounds them, 'yellow' under visible light (x10,000). Under 270-340 nm UV radiation, no blue fluoresence is seen within the cells (not shown here); B, Malignant melanoma cells treated with 1 mg/ml PB-100 as in A. PB-100 entered nuclei and concentrated in nucleoli, as shown by the yellow color of these organelles under visible light; C, Blue fluorescence under 270-340 nm UV radiation (x10,000).

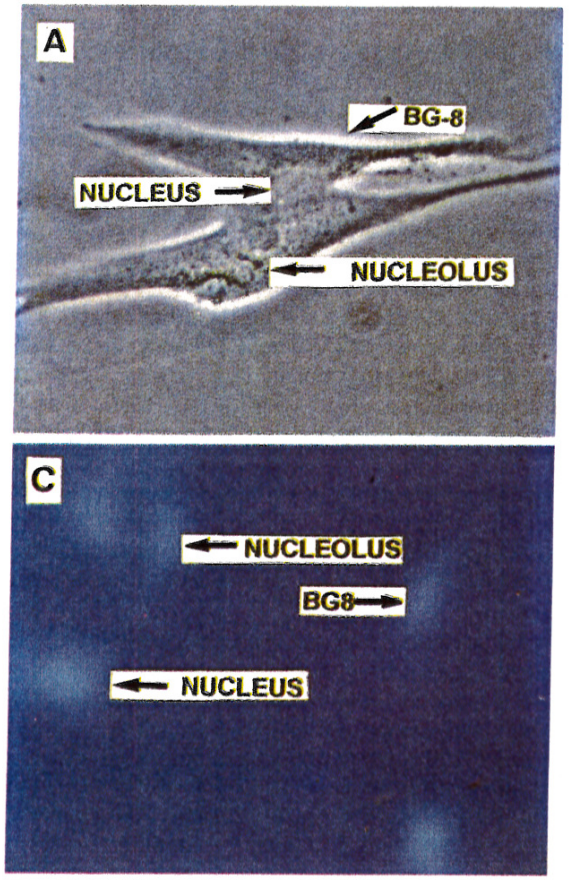

Figure 3. Light and UV microscope observations of BG-8 treated cells. A, Normal fibroblasts treated with 1 mg/ml BG-8 for 10 min at 37°C during growth phase. The anticancer agent remained outside these cells, as shown by the halo that surrounds them, yellow under visible light (x10,000). Under 270-340 nm UV radiation, no blue fluoresence is seen within the cells (not shown here); B, Malignant melanoma cells treated as in A. BG-8 entered cytoplasm and nuclei, both yellow-stained under visible light; C, Under 270-340 nm UV radiation, a blue fluorescence is seen in nuclei, and less brightly in cytoplasm (x10,000).

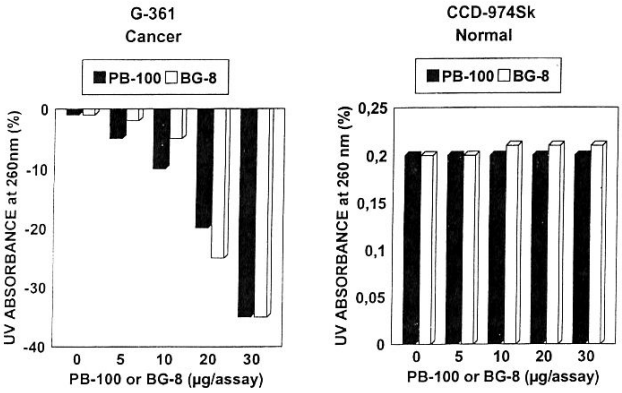

Figure 4. Effect of PB-100 and BG-8 on UV absorbance of human melanoma (G-361) and normal fibroblast (CCD-974Sk) DNAs. Purified DNA was dissolved (10 µg/ml) in Tris-HCl buffer (3 µM), pH 7.3. PB-100 and BG-8 were dissolved in 1 ml buffer, then added to the blank and to the DNA solution. After agitation at 20°C, its UV spectrum was determined. Concentrations inducing maximal increase or decrease of UV absorbance at 260 nm were then measured (expressed as % of control). Data are mean values of three separate determinations.

Table I. Effects of ferritin, $FeCl_3$ $ZnCl_2$, PB-100 and BG-8 on human melanoma (G-361) and human fibroblast (CCD-974Sk) cell lines.

Compounds	% Cell increase or decrease G-361	CCD-974Sk
Ferritin 0 ng/ml	-	-
Ferritin 50 ng/ml	+62.16	+19.53
Ferritin 50 ng/ml + PB-100 100 µg/ml	-63.16	+16.99
$FeCl_3$ 0 µg/ml	-	-
$FeCl_3$ 25 µg/ml	+20.15	+18.76
$FeCl_3$ 25 µg/ml + PB-100 100 µg/ml	-54.73	+21.59
$ZnCl_2$ 0 µg/ml	-	-
$ZnCl_2$ 100 µg/ml	-78.42	+25.60
PB-100 100 µg/ml	-96.61	+5.50
Ferritin 0 ng/ml	-	-
Ferritin 50 ng/ml	+62.16	+19.53
Ferritin 50 ng/ml + BG-8 100 µg/ml	-70.19	+19.11
$FeCl_3$ 0 µg/ml	-	-
$FeCl_3$ 25 µg/ml	+20.15	+18.76
$FeCl_3$ 25 µg/ml + BG-8 100 µg/ml	+26.25	+17.08
$ZnCl_2$ 0 µg/ml	-	-
$ZnCl_2$ 100 µg/ml	-78.42	+25.60
BG-8 100 µg/ml	-83.16	+6.22

Human melanoma (G-361) and human fibroblast (CCD-974Sk) cell lines were grown for 48 h at 37°C in the absence and presence of ferritin, $FeCl_3$ and $ZnCl_2$, either used alone (controls) or added prior to PB-100 or BG-8. Cells were then trypsinized and counted with a Coulter counter. Data are mean values of three independent experiments (expressed as % of variation).

presence of ng/ml concentrations of ferritin, melanoma cell multiplication dose-dependently increases, and 100 ng/ml ferritin enhance it by 80%. The same ferritin dose only induces a 20-25% increase of fibroblast multiplication. $FeCl_3$ at a 25 µg/ml concentration increases both melanoma cell and fibroblast multiplication by about 20% (Table I).

In the presence of ferritin or $FeCl_3$, PB-100 still selectively destroys melanoma cells, but its activity decreases, respectively, by about 30% and 40%. Ferritin reduces BG-8 antiproliferative activity by about 10%. In contrast, $FeCl_3$ (25 µg/ml) renders BG-8 inactive (Table I).

Selective inhibition of melanoma cell proliferation by $ZnCl_2$. We had previously shown that $ZnCl_2$ dose-dependently induced a significant decrease (up to 75%) of glioblastoma cell *in vitro* multiplication, while, on the contrary, it slightly enhanced normal astrocyte multiplication (9). In the present study, Zn^{2+} ions also dose-dependently decreased melanoma cell proliferation (by up to 80%, using 100 µg/ml $ZnCl_2$), and progressively enhanced fibroblast multiplication (by up to 20-25%, using 100 µg/ml $ZnCl_2$) (Table I). Under the same experimental conditions, $ZnCl_2$ does not compete with the anticancer activity of either PB-100 or BG-8 (data not shown).

Discussion

After several decades of anticancer research, the most widespread drawback of chemotherapy still is its lack of selectivity for malignant cells, coupled to toxicity for rapidly multiplying normal cells, blood cells being particularly vulnerable targets (3,13,14). Another major problem, drug resistance, intrinsic or acquired, also remains unsolved in spite of extensive investigations (15-19); it is notably manifest in about 80% of solid tumors (20). According to a recently expressed opinion, 'it appears that nonspecific cytotoxic chemotherapy drugs will never be effective in the treatment of the common solid tumors' (21).

Melanoma is a particularly challenging, highly metastatic, steadily increasing type of tumor (22,23). When past an early stage amenable to surgery, and at metastatic stages, single agent or combination chemotherapy is commonly used (24). Its efficacy is seriously limited, ranging from 24% for the presently most active single agent (DTIC) to not more than 50% for various combination therapies, which often prove more toxic. Moreover, response to these various treatments is not durable (23). Other solutions have been proposed, such as immunochemotherapy (25,26) or use of the natural compound betulinic acid, selectively active on melanoma cells and inactive on normal cells, but also inactive on other types of cancer (23).

As an outcome of long series of experiments in which we compared DNAs from numerous cancer cell types and their normal counterparts, we were able to demonstrate, over 15 years ago, that cancer DNA differs from normal by the fact that it is a destabilized molecule. Its 260 nm UV absorbance is constantly higher than that of its normal counterpart. This cancer DNA hyperchromicity indicates that its molecule contains multiple broken H-bonds, and, as a consequence,

extended areas where its strands remain permanently separated. This facilitates DNA binding of multiple exogenous or endogenous molecules, many of which increase H-bond breakage, strand separation and DNA destabilization: such molecules are carcinogens. As a result of their separation, isolated DNA strands of the cancer DNA molecule become permanently accessible to replication and transcription enzymes, especially now that normally unused initiation sites have become available on the newly exposed strand areas. We showed that cancer DNA hyperchromicity clearly correlates with its increased replication and enhanced cancer cell multiplication. In addition, expression of genes exposed on separated strand areas leads to abnormal protein syntheses, inducing formation of molecules specific to the cancer cell and on which the latter comes to depend. In contrast, normal cell DNA strands only separate locally and transiently during replication and transcription; they are thus much less accessible to DNA-binding molecules (8-11).

Based on these findings, we devised a rapid and simple screening test, the Oncotest (27), which enabled us not only to identify carcinogenic substances, but also several plant-derived molecules, such as PB-100 and BG-8, which selectively 'restabilize' cancer DNA, bringing its separated strands back together in a non physiological manner, but do not affect normal (noncancer) DNA. The anticancer activity of such molecules is evidenced by the following: they dose-dependently decrease cancer DNA hyperchromicity, by binding to the 'open' cancer DNA chains, with which they form a noncovalent complex (5,10,28); cancer DNA replication and cancer cell multiplication decrease, while the supply of cancer specific proteins is shut off, leading to cancer cell death. In contrast, these anticancer agents have no effect on normal DNA UV absorbance and replication, because they do not bind to its 'closed' chains, and thus do not modify normal cell multiplication. In this way, they are not toxic for non malignant cells. The selectivity for cancer DNA and the efficacy of PB-100 and BG-8 was demonstrated *in vitro* and *in vivo* (29,30).

Selectivity of PB-100 and BG-8 is additionally supported by our recent *in vitro* microscopic observations that PB-100 and BG-8 enter malignant cells and rapidly come to concentrate in the nucleus, whilst they do not enter normal cells at all. Observations on melanoma cells and normal fibroblasts fully confirm data obtained with glioblastoma cells and normal astrocytes (12). It is logical to postulate that, in the first place, the possibility for the two anticancer agents to enter the malignant cell and impossibility to penetrate the normal cell depend on the long-known fact that tumor and nontumor cell membranes differ from each other (31). This point is being further investigated. As concerns anticancer agent nuclear localization, we had demonstrated that PB-100 and BG-8 selectively bind to purine bases (12). Purine-rich stretches appear to be more easily available in cancer DNAs (32). They are also found in DNA and RNA contained in nucleoli, where PB-100 concentrates.

PB-100 (flavopereirine) and BG-8 (alstonine) are similar beta-carboline alkaloids, but the BG-8 molecule is larger, containing an additional cycle (5), and although both molecules have a negatively charged and a positively charged nitrogen, molecular differences induce these atoms to behave differently. While BG-8 apparently remains biochemically unmodified, PB-100 easily converts from the bipolar to the unipolar form in which the N^- easily binds to some molecule in the environment and only the N^+ remains unoccupied and active. Size difference and biochemical modifications may account for the slight discrepancies in the behaviours of the two anticancer agents. We are at present investigating why BG-8 partially localizes in the cancer cell cytoplasm, whilst PB-100 apparently does not remain there, but goes directly to the nucleus.

Trace metals have multifunctional, central biological roles and are both influenced by, and active on, malignant conditions, which may dangerously increase their levels. This is why we assayed iron and ferritin on our cell lines at concentrations which, though above physiological levels, are within the range of those encountered in malignancies. Iron ions, present in all body tissues, are required for cell growth and multiplication and were found to accelerate *in vitro* malignant cell proliferation (33,34). Data from the present experiments show that ferric ions dose-dependently and slightly enhance, to an identical extent, melanoma and fibroblast multiplication, by up to about 20% using 25 µg/ml $FeCl_3$. In contrast, ferritin differentially and dose dependently stimulates cancer and normal *in vitro* cell proliferation: at a maximum ferritin concentration of 100 ng/ml, with respective increases of 80% and 20%, G-361 melanoma cell multiplication becomes four-fold that of CCD-974Sk fibroblasts. These data fully confirm those obtained using glioblastoma cells and astrocytes (9). Ferritin, a cancer marker (35), increases in the blood of cancer patients and thus becomes a permanent tumor-enhancing hazard (36); indeed, we had long ago demonstrated its carcinogenic activity (7).

PB-100 activity is differentially affected by the presence of ferritin and $FeCl_3$ (Table I); cancer cell destruction, though less than the 98%-100% observed when the agent is used alone, still reaches respectively 63% and 55%. In contrast, BG-8, almost impervious to the presence of ferritin, with cancer cell destruction at 70%, is however totally inhibited by $FeCl_3$. Neutralization of the anticancer agent's efficacy might be explained by binding of the ferric ion to the negative nitrogen atom of the bipolar BG-8 molecule, whereas, in PB-100, this nitrogen atom is already bound to other biological molecules and thus inaccessible to ferric ions. Although extreme ferritin and iron levels are used in these assays, these various observations, taken together, underscore the importance of the patient's ferritin and iron levels for cancer therapy protocols.

Zinc, another ion central to life processes, was reported to inhibit malignant cell growth, while poorly affecting non malignant cells (37,38) and various explanations were offered for its antiproliferative activity, such as induction of cell lipid peroxidation (38). Our own *in vitro* experiments confirmed the differential, dose dependent activity of zinc on cancer and normal cells, using glioblastoma cells and astrocytes (9), and, in the present study, melanoma cells and fibroblasts. In the presence of 100 µg/ml $ZnCl_2$, melanoma cell proliferation exhibits a notable, 80% decrease, and, on the contrary, fibroblast multiplication experiences a slight, 20% increase (Table I). Zinc antiproliferative activity on

tumor cells does not compete with that of PB-100 or BG-8, suggesting that their binding sites differ. These data confirm that zinc addition may be beneficial to tumor patients.

The selective action and nontoxicity of PB-100 and BG-8 is an asset for cancer therapy, notably for the difficult treatment of melanoma. In addition, activity of these anticancer agents is facilitated by their association with low doses of radiotherapy and antimitotics, just sufficient to enhance H-bond breakage and cancer DNA strand separation (contrary to high doses, which induce chromosome damage), which improves the opportunity of anticancer agent binding to cancer DNA (7). Open clinical trials have indeed confirmed the efficacy of PB-100 and BG-8 in various malignancies (8). The efficacity of PB-100 and BG-8 is confirmed by the experience of a number of physicians who have used these products over the years, and, using their specific fluorescence, have also observed *in vivo* their selective localization in malignant cells (Dr T. Nawrocki, personal communications, Unité d'Antropologie et d'Ecologie médicale, Faculté de Médicine, Paris XIII, France).

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