INTERNATIONAL JOURNAL OF ONCOLOGY 7: 81-85, 1995

The anticancer agent PB-100 concentrates in the nucleus and nucleoli of human glioblastoma cells but does not enter normal astrocytes

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Received March 13, 1995; Accepted May 11, 1995

Abstract. Selectivity of the anticancer agent PB-100 for malignant cells, already demonstrated using cell growth and viability evaluation, is now confirmed by microscopic observations. PB-100 is easily detected inside cells by its yellow color under visible light and by its blue fluorescence; it may be measured in isolated nuclei using its characteristic UV absorbance. After short treatment of human BCNU-resistant glioblastoma cells (U 251) and normal astrocyte controls (CRL 1656), PB-100 accumulates in the malignant cell nucleus, particularly concentrating in the multiple nucleoli and rapidly inducing glioblastoma cell death, whilst, in contrast, the anticancer agent does not even enter normal cells. We had already shown that PB-100 binds to DNA of cancer cells, but not to that of normal cells. *In vitro* tests described in this report indicate that PB-100 binds to purine bases, but not to pyrimidines, of various ribopolymers and its binding to purine rich nucleic acid stretches is inferred.

Introduction

The cell nucleus is a choice target for anticancer drugs, which, besides altering DNA, may inhibit DNA polymerase activity (1-4), bind to nuclear proteins (5-8), interfere with ribosomal RNA processing in the nucleolus (9) or damage the nuclear membrane (10,11). Yet the majority of these anticancer compounds show no selectivity for malignant cells in preference to normal cells. Even the affinity of positively charged anticancer drugs for malignant cells which have a highly negative membrane potential, such as carcinoma cells, does not prevent these molecules from harming similarly charged cardiac cells (11). In contrast, the anticancer compound PB-100 exhibits high selectivity for cancer cells (12). We recently demonstrated that, *in vitro*, concentrations which kill human BCNU resistant U 251 glioblastoma cells do not affect normal CRL 1656 astrocytes (13).

The present study demonstrates that PB-100 rapidly enters the malignant cells, where it accumulates in the nucleus and particularly in the nucleoli; but it does not enter normal cells.

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; PB-100, flavopereirine; 4-GMP, guanylic acid oligomer; tRNA, transfer RNA

Key words: astrocytes, glioblastoma cells, PB-100 alkaloid, nucleus, nucleolus

Materials and methods

Chemicals. 5'-polyadenylic acid (poly-A), 5'-polycytidylic acid (poly-C), 5'-polyguanylic acid (poly-G), 5'-polyuridylic acid (poly-U), 4-GMP, GDP: Sigma Chemical Co.,St Louis, MO, USA. Trypsin, RPMI 1640 culture medium, fetal calf serum, glutamine: Gibco, Grand Island, NY, USA. All other chemicals (grade A): Prolabo, France.

DNAs from U 251 and CRL 1656 cells were isolated as described elsewhere (12,14). The alcaloid PB-100 was purified in our laboratory (12).

Cell lines and culture techniques. The U 251 human BCNU resistant glioblastoma cell line was obtained from the Swedish Cell Collection, Uppsala, Sweden and the CRL 1656 Mpf normal astrocyte line, from the American Type Culture Collection, Rockville, MD, USA. Cells were grown at 37°C on RPMI 1640 medium containing 10% fetal calf serum. Absence of mycoplasma and bacteria was checked every 3 months. Both cell types were cultured in 6-well tissue culture plates (9.8 cm^2 wells), starting from a $4x10^4$ cell inoculum.

Short treatment of glioblastoma cells and astrocytes with PB-100. Cells were grown for 14 h and 1 mg/ml PB-100 (sterilized distilled water solution) was then added. Incubation lasted 5, 10, 20, 25 or 30 min. Aliquots were removed for further culture of treated cells after dilution with fresh medium and for morphological studies of both treated cells and untreated controls.

Microscopic examination of untreated and PB-100 treated cells. Cells were observed with an inverted microscope, under either visible light or UV (270-380 nm).

Microphotographs were taken with Panther Professional color film (1600 ASA); exposure: 5-10 sec.

Extraction and recovery of PB-100 from malignant and normal cell nuclei. 10^4 cells of each type were used. (i) Isolation of nuclei: 1 ml Nonidet was added to 20 ml of cells. Mixture was ground for 2 min in a Potter prior to centrifugation at 500 g for 10 min. Pellet was dissolved in 1 ml PBS X, then centrifuged at 500 g for 10 min. Supernatant was collected for spectroscopic determinations. (ii) Recovery of PB-100 from nuclei: Nuclear pellets were suspended in 1 ml PBS X; 2 ml lauryl sulphate, 1 ml HCl 6 N, 2 ml chloroform and 2 ml distilled water were added. Mixture was agitated for 5 min, then centrifuged at 500 g for 10 min. Extraction procedure was repeated, chloroform phases were mixed and evaporated to dryness. The residue, dissolved in 1 ml distilled water, was used for UV absorbance determinations (at 252 nm). (iii) Experiments using isolated nuclei: Nuclei were suspended in 1 ml PBS X and incubated for 2 h with 300 µg PB-100. When incubation was terminated, nuclei were centrifuged at 500 g for 10 min, pellets were washed with, resuspended and processed as in (ii).

Results

Morphological studies. (i) Visible light observations: Incubation with PB-100 (1 mg/ml) ranging from 5 to 30 min (see Materials and methods) does not modify normal CRL 1656 astrocyte proliferation and induces no morphological changes in cells. A yellow cloud of PB-100 surrounds cell contours, but does not cross the cell membrane (Fig. 1A).

In contrast, after a 10 min incubation with PB-100, nuclei of U 251 glioblastoma cells have already taken on a yellowish hue in places and nucleoli have clearly begun staining light gold, indicating local accumulation of the anticancer agent, whilst no stain is apparent in cytoplasm and cytoplasmic organelles. Nuclear and nucleolar staining increases with time and, after a 20 to 30 min incubation, over 98% of PB-100 treated glioblastoma cells, which have yellow nuclei and orange nucleoli, both enlarged, are agglutinated and killed. Fig. 1B depicts results of a 30 min incubation with PB-100. These observations indicate that PB-100 rapidly and selectively penetrates into the malignant cell nucleus and specially concentrates in nucleoli (Fig. 1B).

(ii) Fluorescence observations: Whether untreated or treated with PB-100, contents of normal astrocytes do not exhibit any fluorescence.

In contrast, the same nuclei of treated glioblastoma cells which had stained yellow under visible light now exhibit a blue fluorescence, which is particularly striking in nucleoli (Fig. 2B, cf. Fig. 2A) and is the color of PB-100 under 270-380 nm UV illumination. Cytoplasm is not stained, indicating that it contains no detectable amounts of PB-100.

Recovery of PB-100 from cell nuclei. Following extraction of the anticancer agent from nuclei which had been either directly isolated from cells or incubated with PB-100 after isolation (see Materials and methods), both chromatography and measurement of characteristic PB-100 252 nm UV absorbance confirmed the presence of the agent in

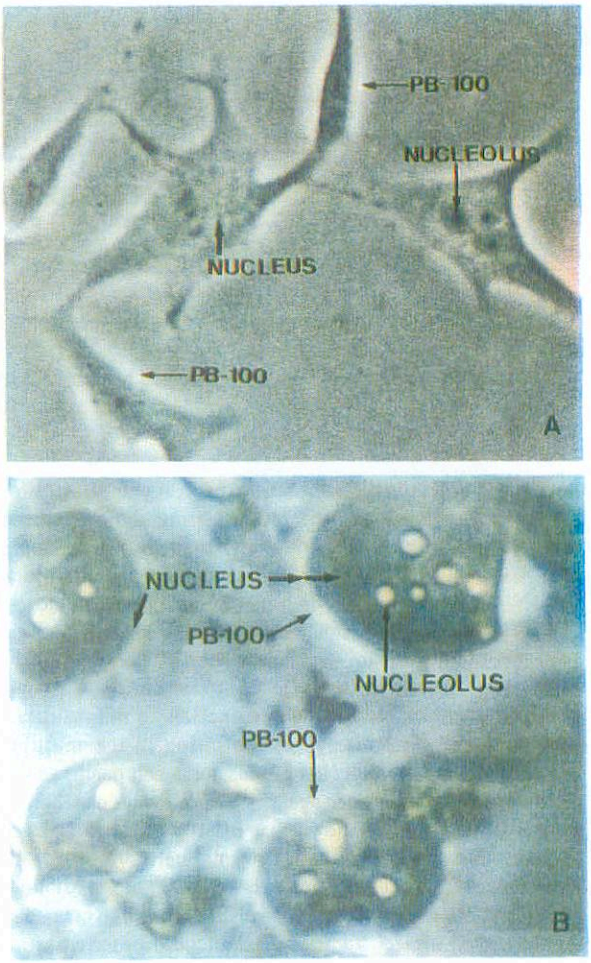

Figure 1. A: Normal astrocytes treated with PB-100. Normal CRL 1656 astrocytes were treated for 30 min with 1 mg PB-100/ml during growth phase. PB-100 did not enter these cells, but remained outside and around them, as shown by the yellow halo under visible light (x5000). B: Human BCNU resistant glioblastoma cells treated with PB-100. U 251 human glioblastoma cells were treated for 30 min with 1 mg PB-100/ml during growth phase. PB-100 entered nuclei and concentrated highly in the nucleoli, as shown by their yellow color under visible light (x25000).

glioblastoma cell nuclei and its absence from astrocyte nuclei (Fig. 3).

In vitro binding of PB-100 to various nucleic acid bases. In view of the nuclear and nucleolar targeting of PB-100, we tested its ability to bind to various polymers of nucleic acid bases. It may be seen from Fig. 4 that PB-100 binds to poly A and poly G, but not to poly C. However, it binds weakly to poly U, absorbance (hypochromicity), indicating that the anticancer agent binds to single strand oligo G. However, it does not bind to GDP.

Fig. 5 indicates the effect of PB-100 on 260 nm UV absorbance of DNAs isolated from normal CRL 1656 astrocytes and BCNU resistant U251 glioblastoma cells. PB-

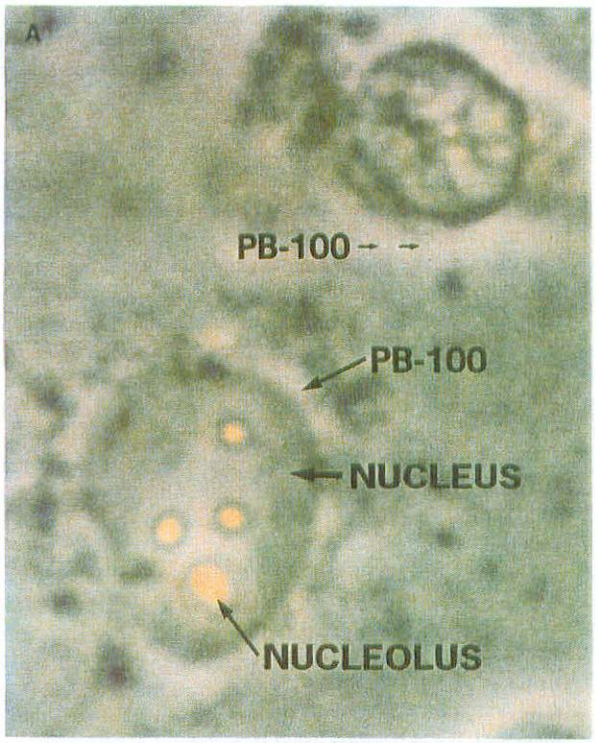

Figure 2. Magnified nucleus from a PB-100 treated glioblastoma cell. A: Nucleus from a glioblastoma cell treated for 30 min with 1 mg PB-100/ml during growth phase. PB-100 concentrated in the five nucleoli, as evidenced by their marked yellow-orange color under visible light. B: the same nucleus, under 270-340 nm UV light.

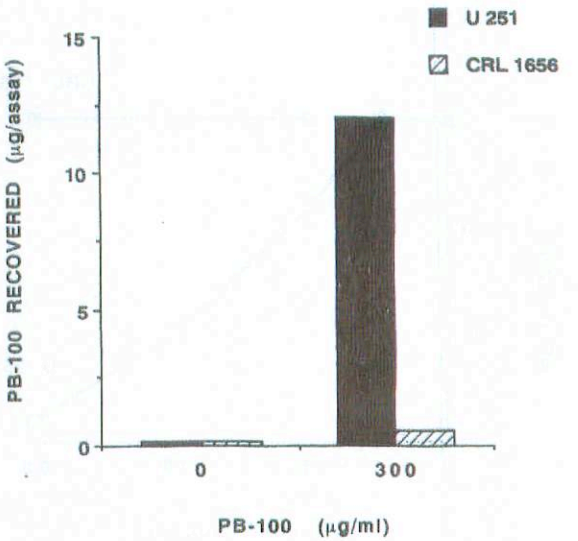

Figure 3. Recovery of PB-100 from nuclei of treated cells. Glioblastoma cells and normal astrocytes were treated for 30 min with 300 µg PB-100/ml. For isolation of nuclei and recovery of PB-100, see Materials and methods.

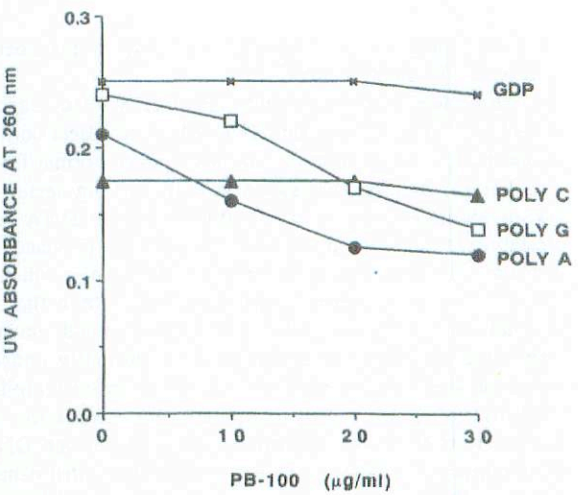

Figure 4. *In vitro* binding of PB-100 to purine and pyrimidine polymers. Each polymer was dissolved in Tris-HCl buffer (10^{-3} M, pH 7.3). Solution was gently agitated at 20°C. A blank solution containing PB-100 was prepared. PB-100 concentrations inducing maximal decrease of polymer solution 260 nm UV absorbance were determined (three separate determinations).

100 does not bind to the normal cell DNA, but forms a complex with the malignant cell DNA, inducing a marked decrease of UV absorbance (hypochromicity).

Discussion

Many years of our research have already been devoted to selecting and investigating highly selective anticancer drugs

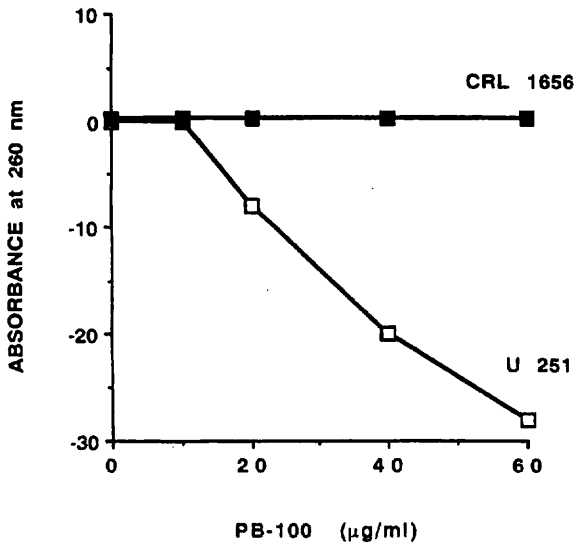

Figure 5. Effect of PB-100 on 260 nm UV absorbance of glioblastoma cell and astrocyte DNAs. UV absorbance of malignant and normal cell DNAs was measured as described in legend to Fig. 3.

of natural origin, one of which is the plant derived beta-carboline alkaloid PB-100 (15-17).

We showed that these agents specifically attack cancer cells by binding to malignant DNA, but do not affect normal (noncancer) cells because they do not bind to normal DNA (12,13,18). Briefly, the reason is that, as long series of experiments enabled us to establish, cancer DNA is a destabilized molecule: many of its H-bonds are permanently broken, as evidenced by its 260 nm UV absorbance, which is always markedly higher (hyperchromicity) than that of normal DNA. This means that cancer DNA strands remain separated over large areas, where new, normally unused initiation sites become exposed, accounting for uncontrolled cell multiplication and dysregulated gene expression. In support of this, we demonstrated that cancer DNA hyperchromicity closely matches increased *in vitro* cancer DNA replication and malignant cell multiplication (18). The 'open' cancer DNA chains are easily accessible to many exogenous and endogenous molecules. In contrast, normal DNA strands only separate locally and transiently for replication and for gene expression and these 'closed' chains are much less accessible to DNA binding agents.

Using our specific *in vitro* test (Oncotest) (15,18), we were then able to find a number of agents having the unique property of binding to the open tumor DNA chains and bringing separated strands back together, whilst not binding to the normal DNA closed chains (12).

We have recently reported (12,13) on the effect of PB-100 on a cell line (U 251) derived from the highly malignant and frequent human brain tumor, glioblastoma, using a normal human astrocyte line (CRL 1656) as its non-cancer control counterpart. The present work is a follow-up to previous *in vitro* research which had demonstrated that PB-100 was active on glioblastoma DNA, but not on astrocyte DNA (Fig. 5) and that the same PB-100 concentrations which killed 98-100% glioblastoma cells did not affect astrocyte multiplication (12).

Microscopic observations, confirmed by spectroscopic analysis, now demonstrate that PB-100 rapidly enters the glioblastoma cells and straight away accumulates in the nucleus, where it particularly concentrates in the nucleoli. Both these organelles take on the characteristic color of PB-100, golden yellow under visible light and blue under UV radiation. Cytoplasm is not stained, indicating that the agent moves directly to the nucleus. Nuclear and nucleolar staining occurs within minutes, increases with time and culminates when malignant cells, exhibiting enlarged nuclei and nucleoli, agglutinate and die (Figs. 1B and 2B).

Parallel experiments using normal astrocytes show that PB-100 does not even enter these cells, which, under visible light, simply remain surrounded by a yellow cloud of the agent (Fig. 1A).

The presence of PB-100 in glioblastoma cell nuclei and its absence from astrocyte nuclei, was confirmed after extraction of the agent from these organelles and spectroscopic determinations using the characteristic UV absorbance of PB-100 at 252 nm. This was further corroborated by experiments involving incubation of isolated nuclei with the anticancer agent (Fig. 3).

The cell nucleus is a common target for many anticancer drugs; while DNA itself may be directly affected, for instance by intercalating agents, molecules like daunomycin derivatives may inhibit DNA polymerase activity (2), or others, like CCNU, bind to nuclear proteins (7). Nucleoli, which develop from DNA regions encoding precursor ribosomal RNA (pre-rRNA), known as nucleolar organizers, contain large amounts of precursor rRNA and ribonucleoproteins and are the site of extensive rRNA transcription, processing and protein addition in view of ribosome formation (19,20). Some anticancer drugs, such as ethidium and ellipticine, were found to inhibit nucleolar processing of 45 s precursor rRNA in L1210 mouse lymphoma cells (9).

From our *in vitro* investigations of PB-100 interaction with purine and pyrimidine polymers, it may be seen that PB-100 preferentially binds to purine bases. Within the nucleus, it may be expected to bind to A and G rich stretches present in nuclear DNA (21) and to some transfer RNAs which are known to contain self complementary loops comprising adjacent A or G nucleotides; inside the nucleolus, it might bind to purine-rich stretches in DNA pre-rRNA genes, in precursor ribosomal RNA and in ribonucleoproteins.

PB-100 induced nuclear and nucleolar enlargement might then be interpreted as arising from inhibition or disturbance of various processing paths, possibly through warping of enzyme synthesis, followed by accumulation of abnormal and/or unprocessed molecules. This would result in part from the fact that, by binding to and bringing together, isolated strands of malignant DNA, carrying normally unexposed initiation sites, PB-100 not only inhibits cancer DNA replication, but also shuts off the supply of malignancy-specific RNA and proteins on which the cancer cell had come to be dependent. PB-100 binding to RNA stretches would enhance these disturbances. The practical,

experimentally demonstrated consequence is death of malignant cells.

Is nuclear targeting the sole cause of PB-100 selectivity for cancer cells? On microphotographs, the anticancer agent is seen, in the first place, to selectively enter the malignant glioblastoma cells and be prevented from entering the normal astrocytes. Should this differential behavior be considered as a separate process from nuclear interaction?

Cases have been described (10,11) in which selective entry of anticancer drugs into malignant cells depended on electric charge interactions: drug molecules which are positively-charged at physiological pH preferentially accumulate in cancer cells that have highly negative membrane potentials, as, for instance, carcinoma cells. Unfortunately, normal cardiac muscle cells also have a pronounced negative membrane potential, accounting for the cardiac toxicity of anthracyclines such as adriamycin (11). Yet, in these investigations, positively-charged, neutral or zwitterionic compounds exhibited no selectivity of this kind (11,12).

PB-100 can behave as a zwitterion because of its quaternary nitrogen. Moreover, its smallish size (MW: 247) should facilitate passive diffusion accross cell membranes, whether malignant or normal. Yet the experiments we report herein, as well as previous studies (12), seem in favor of a selective intake. Other investigations are underway. It is well known that tumor cell membranes differ from those of normal ones and these properties, of course, ultimately depend on expression of tumor specific genes. It may be inferred that exposure of normally unused initiation sites on isolated cancer DNA strand areas could be responsible for this.

Though many points remain to be investigated, the present study confirm the high selectivity of PB-100 for malignant glioblastoma cells and its innocuity for normal astrocytes. On the basis of these results, taken together with those we previously reported (12,13), as well as the ability of the PB-100 to cross the blood-brain barrier, it may be hoped that this anticancer agent will find its place in therapy of brain malignancies and other cancers.

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