Targeting of Tumor Cells and DNA by a Chlorambucil-Spermidine Conjugate


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ABSTRACT

Many tumor cells, including murine ADJ/PC6 plasmacytoma cells, possess an active energy dependent polyamine uptake system which selectively accumulates endogenous polyamines and structurally related compounds. We have attempted to target the cytotoxic drug chlorambucil to a tumor possessing this uptake system by conjugating it to the polyamine spermidine. Furthermore, since polyamines have a high affinity for DNA, the attachment of spermidine to chlorambucil should also facilitate its targeting to DNA. This was supported by the observation that the chlorambucil-spermidine conjugate was approximately 10,000-fold more active than chlorambucil at forming interstrand cross-links with naked DNA. In vitro cytotoxicity and in vivo antitumor studies were carried out using the ADJ/PC6 plasmacytoma. In vitro, using [3H]thymidine incorporation to assess cell viability following a 1-h exposure to control and polyamine depleted ADJ/PC6 cells, chlorambucil-spermidine was 35- and 225-fold, respectively, more toxic than chlorambucil. The increased toxicity of the conjugate compared to chlorambucil was possibly due to enhanced DNA binding and/or facilitated uptake via the polyamine uptake system. The enhanced toxicity of the conjugate but not chlorambucil by prior polyamine depletion with difluoromethylornithine, together with the observation that the conjugate but not chlorambucil competitively inhibited spermidine uptake into tumor cells, supported the suggestion that the conjugate utilized the polyamine uptake system. In vivo following a single i.p. dose, the conjugate was 4-fold more potent than chlorambucil in its ability to inhibit ADJ/PC6 tumor growth in BALB/c mice. However, the therapeutic index was not increased.

Our results support the hypothesis that polyamines linked to cytotoxics facilitate their entry into tumor cells possessing a polyamine uptake system and increase their selectivity to DNA. This may have therapeutic application in the delivery of cytotoxic agents linked to polyamines to certain tumors.

INTRODUCTION

Polyamines are ubiquitous low molecular weight organic cations, which are required for growth and differentiation (1). While the majority of mammalian cells synthesize their own polyamines, a number also possess a specific active transport system for polyamine uptake. The characteristics of polyamine transport in mammalian cells have recently been reviewed (2). A similar uptake system is present in a number of tumor cells including chronic lymphocytic leukemia, lymphomas, and ovarian carcinoma. It is taken up by various tumor cells including chronic lymphocytic leukemic lymphocytes, and simple diffusion following which it exerts its cytotoxic effects by interacting with DNA (18). Cells in early G1 or M phases of the cell cycle are generally most sensitive to nitrogen mustards (19). The common dose limiting toxicity of polyamine analogues is dependent on the availability of terminal amino groups (10). Also, for diamines, transport specificity is greatest for those having chain lengths similar to that of spermidine and least for those similar to that of putrescine (11). Among triamines, transport is greatest for those with a chain length similar to those of spermidine and spermine with an optimal spacing of four carbon atoms separating two positively charged nitrogen atoms (11).

Recently the uptake characteristics of a number of mammalian cell lines possessing the polyamine uptake system have been studied under identical experimental conditions and considerable differences in the kinetic parameters describing polyamine uptake have been observed (12). It has been suggested that in some tissues or cells more than one carrier mechanism may exist for polyamines. For example, in isolated rat enterocytes a separate mechanism for spermidine and spermine uptake from that for putrescine has been reported (13). Other cells reported to have more than one transporter are bovine adrenocortical, Chinese hamster ovary, and B16 melanoma cells (2). We wish to exploit differences in the uptake systems of normal and tumor cells in order to selectively increase uptake into tumor cells. This partly based on the rationale that tumor cells may have a higher affinity for polyamines than normal tissues and also that there are different structural requirements for compounds to be accumulated by normal and tumor cells. Increased putrescine uptake occurs in transformed baby hamster kidney cells compared to nontransformed cells (14), in undifferentiated mouse neuroblastoma cells compared to differentiated cells (15), and in a rat gliosarcoma compared to normal surrounding brain (16).

Some antitumor agents, e.g., MGBG, are known to be accumulated by the polyamine uptake system (7, 9). MGBG is accumulated due to its structural similarity to the polyamine spermidine. Intracellular polyamines may be depleted by pretreatment of cells with DFMO, an irreversible inhibitor of ornithine decarboxylase, the initial rate limiting enzyme in polyamine biosynthesis (1, 17). In order to compensate for the depletion of intracellular polyamines, many cells try to restore levels by increasing the uptake of extracellular polyamines as well as structurally related compounds such as MGBG resulting in intracellular concentrations over 1000-fold greater than extracellular concentrations (7).

Chlorambucil is one of the best tolerated p.o. alkylating agents and is widely used in the treatment of chronic lymphocytic leukemia, lymphomas, and ovarian carcinoma. It is taken up by various tumor cells including chronic lymphocytic leukemic lymphocytes, by simple diffusion following which it exerts its cytotoxic effects by interacting with DNA (18). Cells in early G1 or M phases of the cell cycle are generally most sensitive to nitrogen mustards (19). The common dose limiting toxicity of...
the compound is irreversible bone marrow damage leading to myelosuppression.

In this study, we attempt to selectively target chlorambucil to tumor cells possessing the polyamine uptake system by conjugating the drug to the central nitrogen of spermidine (Fig. 1). This conjugation should result in the active accumulation of the compound by the polyamine uptake system since spermidine substituted on the central nitrogen should be a good substrate for the uptake system. This was demonstrated by Porter et al. (10) who showed that the spermidine molecule can be extensively modified at the N4 position and still be accumulated by the polyamine uptake system. Conjugation of chlorambucil to spermidine should increase both its accumulation and its cell type specificity thus reducing host toxicity. Polyamine depletion with DFMO may further increase accumulation. We propose that in addition to this, conjugation with polyamines may also selectively target the DNA itself due to the polycationic nature of spermidine (20) which gives it a high affinity for DNA (20, 21). This concept is similar to a suggestion by Deny (22) that rationally designed DNA intercalating ligands should be suitable carrier molecules for cytotoxics. Targeting DNA should mean both less chance of losing active drug by reactions with other cell components and also possibly a higher number of lethal cross-links leading to increased drug potency.

In this study we have compared the in vitro and in vivo antitumor activity of chlorambucil and a CLB-SPD conjugate using the ADJ/PC6 plasmacytoma which we have shown to possess a polyamine uptake system. This cell line was chosen due to its sensitivity to alkylating agents (23) and its ability to grow both in vivo and in vitro. The affinity of CLB-SPD for the polyamine uptake system was also investigated by determining its ability to inhibit spermidine uptake. In addition, the ability of CLB-SPD to form interstrand cross-links with naked DNA was also investigated. Our results show that in the ADJ/PC6 plasmacytoma, the CLB-SPD conjugate had a high affinity for the polyamine uptake system. It was also approximately 10,000-fold more effective than chlorambucil at forming interstrand cross-links with naked DNA. The full potential of these in vitro results were not realized in initial in vivo studies.

MATERIALS AND METHODS

Chemicals. [3H]Thymidine (26 Ci/mmol) and [14C]spermidine (117 mCi/mmol) were purchased from Amersham International (Amer-

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for 72 h in the presence or absence of DFMO (2.0 mM) using an initial density of 1 x 10^6 cells/ml. Prior to the toxicity studies, tumor cells were diluted in medium to give a density of 2 x 10^3/ml and aliquots (0.5 ml) were added to culture tubes. Chlorambucil, CLB-SPD (0-0.5 μM), and MGBG (0-10 μM) were added to give a final volume of 1 ml and incubated for 72 h. This 72-h incubation time was chosen in order to allow DNA damage to be manifested as cell death. [3H]Thymidine (0.5 μCi) was added to each tube for the final 2 h of the incubation. In order to compare the effect of a shorter exposure time on the toxicities of the compounds, cells were exposed to chlorambucil (0-40 μM) and CLB-SPD (0-0.5 μM) for 1 h, the medium was replaced, and the cells were incubated for a further 71 h. In addition the effect of spermidine (1, 10, or 20 μM) in the incubation mixture was investigated to determine whether it could inhibit the toxicity of the conjugate by inhibiting its uptake. The effect of polyamine depletion with DFMO was also studied for this shorter incubation time. [3H]Thymidine was added to the cells for the final 2 h as before. At the end of the incubations, the cells were pelleted by centrifugation (1000 rpm for 5 min) and the supernatant fractions were removed. The cell pellet was washed twice with 1 ml cold 0.9% NaCl containing unlabeled thymidine (1 mM) to remove nonspecifically bound [3H]thymidine. Cold 10% trichloroacetic acid (1 ml) was added to the pellet for 10 min and the precipitate was dissolved in 1 ml NaOH (300 μl) and neutralized with 1 M HCl (300 μl), and radioactivity was determined as described earlier. For each drug concentration, [3H]thymidine incorporation was expressed as percentage of the control value in the absence of drug. IC_{50} was calculated in the presence or absence of DFMO. DFMO controls were used where appropriate.

**In Vivo Antitumor Studies.** In vivo antitumor studies were carried out on the ADJ/PC6 tumor based on a method described previously (26). Small pieces (1 mm^3) of ADJ/PC6 tumor (from a freshly removed tumor) were implanted s.c. in the right flank of female BALB/c mice (8 weeks old). The tumors were allowed to grow for 20 days, when animals with similar size tumors (approximately 1 cm^3) were selected for participation in the antitumor study. Mice were divided into treatment groups (3/group) and dosed with a single i.p. injection of freshly prepared solutions of chlorambucil and CLB-SPD (0.25-4 mg/kg) in 10% ethanolic HCl/90% propylene glycol phosphate buffer. (Dosing solutions were prepared by dissolving the compounds in 0.5 M HCl in ethanol to give 10-fold concentrated solutions, which were then diluted prior to dosing with 0.2 M K_2HPO_4 in 45% propylene glycol.) Control groups of 6 animals were treated either with vehicle alone or received no treatment. The tumor was allowed to grow until day 29, when all animals were sacrificed and the tumors were weighed. The weight of tumors from each treatment group was compared to the vehicle control group, and expressed as a percentage of these. The doses inhibiting tumor growth by 90% were calculated and compared. These studies were carried out within the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia.

**RESULTS**

**Interstrand Cross-Linking of DNA.** The ability of CLB-SPD to introduce interstrand cross-links into linear plasmid DNA (approximately 4300 base pairs) was assessed using an agarose gel technique. Following complete denaturation of the DNA to single-stranded, the presence of an interstrand cross-link results in renaturation to double-stranded DNA on neutral agarose gel electrophoresis. CLB-SPD was highly efficient at producing cross-links which were clearly evident at 0.01 μM (Fig. 2). In contrast, cross-links were observed only with chlorambucil under identical conditions at concentrations greater than 10 μM, with the DNA still less than 50% cross-linked at 100 μM (Fig. 2). Thus CLB-SPD was approximately 10,000-fold more efficient than chlorambucil at forming interstrand cross-links in naked DNA. An equimolar mixture of spermidine and chlorambucil (0.001-100 μM) resulted in no enhancement of cross-linking when compared to chlorambucil alone. In fact at the highest concentration, an inhibition of cross-linking (from 40 to 25%) was observed. Spermidine alone (0.001-100 μM) did not cause any cross-linking of DNA.

**Inhibition of Spermidine Uptake.** ADJ/PC6 plasmacytoma cells possessed a saturable, temperature dependent spermidine uptake system (K_m 0.25 ± 0.03 (SEM) μM; V_max 3.2 ± 0.42 pmol/min/10^6 cells; n = 4). At 4°C, uptake of spermidine was reduced by 95%. Chlorambucil (100 μM) did not inhibit spermidine uptake. However, in contrast, CLB-SPD and MGBG, a control compound known to utilize the polyamine uptake system in other tumor cells such as Ehrlich ascites tumor cells, inhibited competitively the uptake of spermidine in ADJ/PC6 cells (Fig. 3) [K_i 0.8 ± 0.07 (3) and 1.95 μM respectively].

**In Vivo Cytotoxicity.** The toxicities of chlorambucil and CLB-SPD were assessed in ADJ/PC6 cells by their abilities to inhibit [3H]thymidine incorporation into DNA. CLB-SPD was approximately 35-fold more toxic than chlorambucil after a 1-h exposure to the ADJ/PC6 cells as indicated by the IC_{50} values (Table 1). When exposed for 72 h, CLB-SPD was only 7-fold more toxic than chlorambucil (Table 1). DFMO itself gave an
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Fig. 3. Lineweaver-Burk plot to show competitive inhibition of spermidine uptake in ADJ/PC6 plasmacytoma cells by MGBG and CLB-SPD. ADJ/PC6 plasmacytoma cells were incubated with [14C]spermidine alone (•) or in the presence of MGBG (50 μM) (□) or CLB-SPD (2 μM) (▲). Data shown are representative of at least two experiments performed in duplicate.

Table 1 In vitro toxicity of chlorambucil and chlorambucil-spermidine conjugate to ADJ/PC6 cells after 1 or 72 h exposure

Cells were exposed to the compound for either 72 h or 1 h and then replaced with fresh medium for a further 71 h. [3H]Thymidine was added for the last 2 h of the incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control IC50 µM at 1 h</th>
<th>Control IC50 µM at 72 h</th>
<th>DFMO IC50 µM at 1 h</th>
<th>DFMO IC50 µM at 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorambucil</td>
<td>8.9 ± 1.19</td>
<td>0.22 ± 0.02</td>
<td>22.5</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Chlorambucil-spermidine</td>
<td>0.25 ± 0.04</td>
<td>0.03 ± 0.003</td>
<td>0.10 ± 0.016</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*IC50 concentration of compound giving a 50% reduction in [3H]thymidine incorporation. Values are mean ± SEM of at least 3 observations except as noted.

Approximate 50% decrease in [3H]thymidine incorporation compared to control cells. This was taken into account when calculating IC50 values in experiments with DFMO. Polyamine depletion with DFMO increased the toxicity of CLB-SPD (2-fold) and actually decreased the toxicity of chlorambucil for both exposure times. This resulted in a striking 225-fold increase in toxicity of CLB-SPD compared to chlorambucil following a 1-h exposure. The level of polyamine depletion achieved in these experiments resulted in a 2-fold increase in the rate of spermidine uptake when present at a concentration of 0.25 μM over a 1-h time period (results not shown). This is comparable to the IC50 concentration of CLB-SPD to control cells. Addition of spermidine (1, 10, and 20 μM) to the incubation medium of the 1-h exposure experiment reduced the toxicity of CLB-SPD (Fig. 4A) but had no effect on the toxicity of chlorambucil (Fig. 4B). The toxicity of MGBG was enhanced at least 3-fold by polyamine depletion. The IC50 for polyamine depleted cells was 3.5 μM whereas for control cells it was greater than 10 μM.

In Vivo Antitumor Activity. A single i.p. administration of both chlorambucil and CLB-SPD caused a marked dose dependent reduction of tumor weight. The in vivo antitumor activity of CLB-SPD was approximately 4-fold greater than chlorambucil when expressed on a molar basis, as indicated by the doses inhibiting tumor growth by 90% (Table 2). However, in vivo the conjugate had a low 50% lethal dose possibly due to acute nervous system toxicity, and as a consequence, the therapeutic index of the conjugate was slightly less than chlorambucil (Table 2).

DISCUSSION

Particularly striking were the in vitro studies comparing the abilities of chlorambucil and CLB-SPD to produce DNA interstrand cross-links. CLB-SPD was approximately 10,000-fold more effective at inducing cross-links in naked DNA than chlorambucil (Fig. 2). This was a property unique to the conjugate inasmuch as equimolar mixtures of chlorambucil and spermidine did not give enhanced alkylation over chlorambucil alone. In fact inhibition of alkylation was observed. Cationic DNA affinity binders, such as polyamines, have been shown previously to dose dependent inhibit the nitrogen mustard alkylation of guanine N-7 positions in the major groove, the primary sites of base alkylation by such agents (27).

The polycationic nature of spermidine gives it a high affinity for DNA (20, 21). In addition it has recently been shown that

![Fig. 4. Effect of spermidine on the toxicity of CLB-SPD (A) and chlorambucil (B). ADJ/PC6 plasmacytoma cells were exposed to CLB-SPD (•) and chlorambucil (O) alone or in the presence of spermidine (1 μM, □; 10 μM, ▲, and 20 μM, ▼) for 1 h. Cell viability was assessed after a further 71 h incubation in fresh medium by measuring [3H]thymidine incorporation into DNA. Data shown are representative of those obtained in two experiments performed in duplicate.](image-url)
while constrained to remain close to DNA the poly ammonium cations retain a high degree of freedom of motion within the polycation-DNA complex (28). Clearly the conjugation of chlorambucil to spermidine has coupled high affinity with the ability of the bifunctional alkylating group to locate sites suitable for efficient DNA interstrand cross-linking in naked DNA. As initial DNA binding will be dictated by the spermidine moiety, the major site of interstrand cross-linking may not necessarily now be between guanine N-7 sites in the major groove characteristic of nitrogen mustards. The precise location of the alkylation reactions and the relative importance of these lesions to the cytotoxicity of the conjugate remain to be determined and are currently under investigation.

We then extended our studies to determine the effects of CLB-SPD on ADJ/PC6 plasmacytoma cells in vivo and in vitro. These studies demonstrated how conjugation of a polyamine, such as spermidine, to chlorambucil resulted in an increase in toxicity both in vitro and in vivo. This enhanced activity may be due to several reasons such as an increased uptake of the conjugate into the cells via the polyamine uptake system, resulting in higher cellular concentrations than can be achieved by chlorambucil alone, which enters by diffusion (18). In vitro, CLB-SPD was a good competitive inhibitor of spermidine uptake (Fig. 3), with a low $K_s$, indicating that it has a high affinity for the polyamine uptake system. This fact itself does not prove that the conjugate actually enters the cells via the uptake system. However, this together with our observations that polyamine depletion with DFMO increased the in vitro toxicity of the conjugate but not chlorambucil is entirely consistent with the suggestion that CLB-SPD was transported by the polyamine uptake system. In our studies, over a 1-h time period, spermidine uptake was increased 2-fold (results not shown) by polyamine depletion. CLB-SPD toxicity was also increased 2-fold which suggests an increase in uptake similar to that of spermidine, possibly by the same uptake system. In addition, our findings that exogenous spermidine also reduced the toxicity of CLB-SPD (Fig. 3) but not chlorambucil in a concentration-dependent manner, possibly by inhibiting uptake of the conjugate, further supported this hypothesis. When exposed to the ADJ/PC6 cells for 1 h, the CLB-SPD conjugate was 35-fold more toxic than chlorambucil but only 7-fold more toxic after a 72-h exposure (Table 1). This difference in toxicity was the result of a large reduction in the toxicity of chlorambucil compared to the 72-h exposure (Table 1). The shorter 1-h exposure time, which may reflect more closely the exposure time in vivo, should facilitate the uptake of the CLB-SPD conjugate by the polyamine uptake system compared to chlorambucil. Especially striking was the 225-fold increase in toxicity of CLB-SPD compared to chlorambucil following a 1-h exposure to DFMO pretreated cells (Table 1). This increase was due to both an increased toxicity of the CLB-SPD conjugate and a decreased toxicity of chlorambucil. Polyamine depletion by pretreatment of cells with DFMO has been reported to increase or decrease the activity of anticancer agents dependent on both the agent and the cell line, most probably due to effects on DNA conformation (29, 30). Such treatment has resulted in a decreased toxicity of chlorambucil in a number of human adenocarcinoma cell lines (29). The enhanced toxicity of the CLB-SPD conjugate could be due both to an altered conformation of the DNA and to an enhanced uptake. Increased uptake and toxicity of the polyamine analogue MGBG following polyamine depletion has been widely reported (30) and was also confirmed in our studies. Furthermore, we have recently demonstrated increased uptake of the CLB-SPD conjugate following DFMO pretreatment. In addition to the increased uptake, enhanced DNA targeting may also occur following polyamine depletion due to less competition for binding to DNA from endogenous polyamines.

The in vivo antitumor studies showed that the conjugate was 4-fold more potent than chlorambucil against the ADJ/PC6 tumor (Table 2). However, the therapeutic index of the conjugate was not increased since acute CNS toxicity (which has been observed clinically in humans with chlorambucil) was observed leading to a lower 50% lethal dose than chlorambucil. This minor increase in antitumor activity did not reflect the 35-fold increase observed in vitro with control ADJ/PC6 cells or the 10,000-fold increase in reactivity with naked DNA. The reasons for this are unclear and may include several possibilities such as alterations in metabolism, pharmacokinetics, intracellular distribution, DNA binding, and repair. We have previously discussed the critical importance of pharmacokinetics in maximizing therapeutic benefits from active uptake systems (31). Provided that the tumor cells have a lower $K_m$ (i.e., higher affinity) for uptake of the cytotoxic conjugate than normal cells in vivo, slow infusion of the conjugate at concentrations near its $K_m$ for tumor cells should lead to a selective accumulation in the tumor cells. This should result in greater antitumor activity plus a reduction in the acute toxicity observed with a bolus dose, in turn leading to an increased therapeutic index. This approach is currently under investigation with CLB-SPD.

In conclusion, the CLB-SPD conjugate showed greater antitumor activity both in vivo and in vitro than chlorambucil. This was due to increased uptake, increased affinity for DNA, or a combination of the two. A remarkable 10,000-fold increased effectiveness for cross-linking DNA was observed following conjugation of chlorambucil with spermidine. This increased activity has not yet been realized in vivo but the targeting of cytotoxics with polyamines clearly warrants further study.

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