Targeting the Cytotoxicity of Topoisomerase II-directed Epipodophyllotoxins to Tumor Cells in Acidic Environments

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ABSTRACT

The epipodophyllotoxins etoposide and teniposide are probably the most important drugs in the treatment of small cell lung cancer. The drugs are used in maximally tolerated doses, and the toxicity of the drugs precludes significant dose increments. The cellular target is the nuclear enzyme topoisomerase II which, in the presence of these drugs, causes an extensive fragmentation of DNA. The cell kill can be antagonized by distinct drug types. We have demonstrated previously that the intercalating drug aclacinomycin A and the cardioprotecting agent ICRF-187 antagonize the cytotoxicity of etoposide in vitro. We have studied possible ways of using this antagonism as a means of differentially protecting normal tissue. Here we demonstrate that the intercalating agent chloroquine prevents the introduction of topoisomerase II-mediated DNA breaks and thereby antagonizes the cytotoxicity of etoposide. This interaction depends on the extracellular pH. Chloroquine, in contrast to etoposide, is a weak base and therefore does not enter the cell if the extracellular fluid is acidic, as is the case in most solid tumors. We propose that such a pH-dependent drug interaction may be useful in directing topoisomerase II drug effects toward solid tumors. Thus, lowering the extracellular pH (pH,) from neutral (pH, = 7.4) to acidic (pH, = 6.0), [3H]chloroquine accumulation was decreased 5-fold in a human small cell lung cancer cell line, OC-NYH, and in murine leukemia L1210 cells. In parallel, the antagonism exhibited by chloroquine on etoposide cytotoxicity was pH dependent. Thus, no protection by chloroquine was observed at pH, = 6.5, whereas at pH, = 7.4, etoposide cytotoxicity was almost completely antagonized with a 400-fold protection or more than eight doublings of the cell population. This exploitation of antagonist extracellular trapping by acidic pH is a novel model for regulation of anticancer drug effects.

INTRODUCTION

The essential nuclear enzyme topo II allows the separation of intertwined DNA strands by creating a transient, double-stranded break in the DNA backbone. This catalytic cycle of topo II is the target of some of the most successful antitumor agents used today, e.g., etoposide (VP-16), in the treatment of testicular and small cell lung cancer (1). Most often, the drugs are used in maximally tolerated doses, and the toxicity of the drugs precludes significant dose increments, which otherwise might have overcome drug resistance. Obviously, an increased selectivity may be obtained by targeting tumor-specific alterations. However, alterations susceptible to targeting on all tumor cells are disappointingly few. Newell and Tannock (2) suggested that variations in pHi could be exploited for a selective targeting of solid tumors. Thus, solid tumors have been observed to vary from normal tissues (5, 6).

In normal tissues. In contrast, the intracellular pH does not appear to vary from normal tissues (5, 6).

The purpose of our studies has been to generate a therapy model which exploits this acidity by directing the cytotoxicity of etoposide to tissues with low pHi.

MATERIALS AND METHODS

Drugs. For use in the clonogenic assay, daunorubicin (Rhone-Poulenc Rorer) and chloroquine (Sigma Chemical Co.) were dissolved in sterile water (4 mg/ml and 30 mg/ml, respectively). Etoposide (VP-16), teniposide (Bristol-Myers Squibb), and m-AMSA (Parke-Davis) were in solution for infusion at 20, 10, and 50 mg/ml, respectively. All drugs were diluted more than 100-fold with tissue culture medium just prior to use. In vitro studies with purified topo II, VP-16 (Bristol-Myers) and m-AMSA (Parke-Davis) were dissolved in dimethyl sulfoxide and chloroquine (Sigma) in water. [14C]VP-16 (16 Ci/mmol) was a generous gift from Bristol-Myers Squibb (Syracuse, NY); [14C]chloroquine (2.6 Ci/mmol) and [methyl-3H]hydromine (25 Ci/mmol) were from Amersham (Buckinghamshire, United Kingdom).

Cell Lines. The cell lines used were the mouse leukemia L1210 cells and the human small cell lung cancer cell line OC-NYH (7). The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum plus penicillin and streptomycin.

Drug Accumulation. Accumulation of [14C]etoposide and [14C]chloroquine were assessed in OC-NYH and in L1210 cells. Single cell suspensions (1 ml of 5 × 10^6 cells) were incubated with [14C]etoposide (10 μM) or [14C]chloroquine (100–500 μM) at various periods and pH levels at 37°C and subsequently centrifuged at 150 × g for 5 min, washed twice with 10 ml of ice-cold phosphate-buffered saline, solubilized with 0.2 M NaOH, and analyzed for [14C] in a Packard liquid scintillation spectrometer (8). Variations in pHi were obtained by adding HCl to the medium.

Measurement of DNA Single-strand Breaks. Drug-induced DNA breaks in [3H]thymidine-labeled OC-NYH and L1210 cells were quantitated by the alkaline elution filter method, as described in detail (9). Instead of irradiation, the cells were exposed to 100 μM H2O2 for 60 min on ice, corresponding to an irradiation dose of 300 rad as described previously (10). DNA was eluted from the filters with tetrappropyl-ammoniumhydroxide-EDTA (pH 12.1) containing 0.1% sodium dodecyl sulfate at a rate of 0.35 ml/min. Fractions were collected at 20-min intervals for 3 h. The fractions and filters were then processed as described by Kohn (9). DNA single-strand break frequency expressed in rad-equivalents was calculated on the basis of first-order elution kinetics (9).

Clonogenic Assay. Drug toxicity was assessed by colony formation in soft agar with a feeder layer containing sheep RBCs as described previously (11). The cell viability was assessed with dye exclusion in a hemocytometer. Single-cell suspensions in RPMI 1640 supplemented with 10% fetal calf serum (complete medium) were exposed for 1 h to the drugs at 37°C and washed twice with complete medium at 37°C at pH 7.4. Cells (2 × 10^5) were plated to obtain 3000–4000 colonies in the control dishes. The colonies were counted after 3 weeks incubation.

DNA Cleavage Mediated by Purified Topo II. Calf thymus topo II was purified according to a previously described procedure (12, 13). The DNA substrates used were either a 28-base pair double-stranded oligonucleotide (Ref. 15; Fig. 5A) or pH-UC19 plasmid (Fig. 5B). The plasmid DNA was linearized with EcoRI (Boehringer Mannheim, Germany) as described previously (14), and both substrates were end labeled with [α-32P]dATP (American and Schenseaum, Version 2.0 (United States Biochemicals). Topo II was incubated with DNA for 30 min at 30°C in 50 μl of 10 mM Tris-HCl (pH 7.5),

Received 12/31/93; accepted 3/30/94.

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1 Supported by grants from the Danish Cancer Society.

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: topo II, topoisomerase II; pHi, extracellular pH; pH, intracellular pH; m-AMSA, 4'-[9-(acridinyl-amino)ethanethiol]-m-anisidide; VP-16, 4'-demethyllepipodophyllotoxin-9-(4,6-O-ethylidene-b-o-glucopyranoside); etoposide, VP-16-213.

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RESULTS

Chloroquine Antagonizes Etoposide-mediated Cytotoxicity. We have screened a number of membrane-permeable bases for their interaction with etoposide-induced cytotoxicity at neutral and acidic pH. This demonstrated that the antimalarial agent chloroquine in noncytotoxic concentrations antagonizes the cytotoxicity of topo II targeting agents such as etoposide, m-AMSA, daunorubicin, and teniposide. The results with etoposide at pH = 7.4 are shown in Fig. 1. Chloroquine in nontoxic doses antagonizes etoposide cytotoxicity in a concentration-dependent manner, and at the highest chloroquine concentrations (550 μM), there is an almost complete inhibition of etoposide toxicity. Protection is almost 2.5 logs, corresponding to seven doublings of the cell population.

Chloroquine Cellular Accumulation Is Dependent on pH. We then measured the effect of variations in pH on the accumulation of chloroquine in OC-NYH and L1210 cells. A reduction in pH from 7.4 to 6.0 was found to decrease the chloroquine accumulation 5-fold in both cell lines. Fig. 2 shows the pH-dependent accumulation in OC-NYH cells after a 1-h incubation with 500 μM chloroquine. In contrast, the accumulation of [3H]etoposide in OC-NYH cells was not influenced by pH (etoposide accumulation was 14 pmol/10⁶ cells and 13 pmol/10⁶ cells at pH = 7.4 and at pH = 6.3, respectively; mean of two experiments). Accordingly, the combination of chloroquine and etoposide represents the desired model system in which agonist accumulation depends on pH, whereas agonist accumulation does not.

Chloroquine Provides a pH-dependent Modulation of Etoposide Cytotoxicity. The prediction that cell kill in this model will depend on pH was correct as demonstrated in Fig. 3A. In both OC-NYH and L1210 cells, etoposide cytotoxicity is largely prevented by chloroquine at pH 7.4, whereas there is no detectable protection by chloroquine at pH 6.3–6.8 (Table 1; Fig. 3A). Similar effects of chloroquine were seen with teniposide (Fig. 3B). It is noteworthy that we observed a reduced cytotoxicity of etoposide and teniposide at pH 6.3–6.8 as compared to the cytotoxicity at pH 7.4. However, the important finding is that chloroquine exerts a 460-fold protection against etoposide at pH 7.4, while no protection was seen at pH 6.5 (Table 1).

Effect of Chloroquine on Topo II-mediated DNA Breaks. We then studied the effect of chloroquine on etoposide-stimulated, protein-concealed DNA strand breaks in cells. As seen in Fig. 4, addition of chloroquine results in an abrogation of the etoposide-induced formation of DNA breaks at pH 7.4. In accordance with the results in the clonogenic assay and with the chloroquine accumulation data, there is no demonstrable chloroquine antagonism at pH 6.3 (Fig. 4). Finally, we investigated the effect of chloroquine on the cleavage activity of purified topo II. Chloroquine inhibited DNA cleavage obtained with the enzyme alone, and chloroquine also inhibited the DNA cleavage stimulation obtained with different topo II targeting agents, such as etoposide and m-AMSA, in a dose-dependent manner with near complete inhibition at 3 mM (Fig. 5). The results with m-AMSA are shown in Fig. 5A, as this is the drug which stimulates topo II-mediated DNA cleavage to the highest extent on the specific DNA substrate containing a single topo II interaction site (15). The same effect of chloroquine on etoposide-induced cleavages was also demonstrated on a large DNA fragment containing a number of topo II cleavage sites (5B). Accordingly, chloroquine inhibits topo II-mediated DNA cleavage both in cells and in vitro using purified topo II.

DISCUSSION

Solid tumors have been observed to develop an acidic extracellular environment (for reviews, see Refs. 3–6). Measurements of tumor pH have been made by using pH-sensitive microelectrodes, which measure primarily pHₐ, and by using 3¹P NMR spectroscopy, which
nents is either normal or close to normal (median, 7.1–7.2; Ref. 5). Taken together, these results suggest that cells in solid tumors are bathed in an acidic extracellular fluid and that cells within solid tumors are capable of regulating the level of pH\(_e\) to physiological levels, despite lower-than-normal levels of pH\(_j\) (3). Here we have described how the combination of two established therapeutics can result in a selective cytotoxicity in acidic environments while cells in a normal microenvironment are protected. The two drugs included in our model are a neutral agonist which is accumulated in cells in a pH\(_d\)-independent manner and a weak base antagonist which accumulates in cells in a manner dependent on pH\(_j\). The agonist is the topo II-targeting epipodophyllotoxin, etoposide. We have screened a number of membrane-permeable bases for their interaction with etoposide-induced cytotoxicity at neutral and acidic pH\(_j\). We report here that the weak base intercalative antimalarial drug chloroquine is a potent antagonist of etoposide-induced cytotoxicity and that the combination of etoposide and chloroquine exhibits a strong pH\(_j\)-dependent cytotoxicity, representing a therapy model which exploits variations in extracellular pH. The combination of the two compounds may thus direct cytotoxicity to tissues with low pH\(_j\).

There is solid evidence that etoposide, as well as a number of other clinically successful drugs such as daunorubicin and doxorubicin (Adriamycin), are active by inhibiting the rescaling of transient double-stranded breaks in the DNA backbone, so-called cleavable complexes, created by the nuclear enzyme topo II (16, 17). Although the precise cell killing mechanism is unknown, an obligatory step for cytotoxicity of the topo II-targeting agents is an increased appearance of cleavable complexes between DNA and topo II (16). The complex

<table>
<thead>
<tr>
<th>pH(_j)</th>
<th>OC-NYH</th>
<th>Percentage survival</th>
<th>Protection (fold)</th>
<th>L1210</th>
<th>Percentage survival</th>
<th>Protection (fold)</th>
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<tr>
<td>pH(_j) 7.4</td>
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<td>0.1 (0.05–0.3)</td>
<td>0.01</td>
<td>Etoposide + CLQ1</td>
<td>70</td>
<td>40</td>
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<tr>
<td></td>
<td>E + CLQ5</td>
<td>46 (44–52)</td>
<td>640 (150–800)</td>
<td>CLQ1</td>
<td>102</td>
<td>97 (95–101)</td>
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<tr>
<td></td>
<td>CLQ5</td>
<td>740</td>
<td></td>
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</tr>
<tr>
<td>pH(_j) 6.8</td>
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<td>0.02</td>
<td>Etoposide + CLQ1</td>
<td>2</td>
<td>0.02</td>
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<tr>
<td></td>
<td>E + CLQ5</td>
<td>6 (4–8)</td>
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<td>CLQ1</td>
<td>97</td>
<td>95 (95–101)</td>
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<tr>
<td></td>
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<td>0.8</td>
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<tr>
<td>pH(_j) 6.3</td>
<td>Etoposide</td>
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<td>1</td>
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<td></td>
<td>E + CLQ5</td>
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<td></td>
<td>E + CLQ5</td>
<td>7 (6–9)</td>
<td>2</td>
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*Drug exposure for 1 h; etoposide concentration was 20 \(\mu\)M in all experiments. Parentheses, range. E + CLQ1, etoposide + 150 \(\mu\)M chloroquine; E + CLQ5, etoposide + 500 \(\mu\)M chloroquine.*

measures predominantly pH\(_j\). Microelectrode measurements have demonstrated that pH\(_j\) is, on average, 0.5 pH units lower in solid tumors (median, 6.9–7.0) than in normal tissues (median, 7.4–7.5; Refs. 5 and 6). By contrast, \(\text{\textsuperscript{31}P}\) NMR studies have shown that pH\(_j\) in tumors is either normal or close to normal (median, 7.1–7.2; Ref. 5).
mechanism of cell kill is susceptible to drug modulation. By inhibiting the initial DNA binding step of the enzyme, it is possible to suppress the interaction between the enzyme, the topo II-targeting drug, and DNA. Our interest in this type of regulation of drug effect was raised by the finding that the clinically active DNA intercalating drug, aclacinomycin, completely antagonized the cytotoxicity of topo II-targeting agents such as etoposide, teniposide, m-AMSA, daunorubicin, and oxazaphosphorin (14, 18, 19). Not only aclacinomycin but also several other DNA binding agents such as ethidium bromide (20) and 9-aminoacridines (21, 22) can antagonize the cytotoxicity of topo II-targeting agents. Investigations have demonstrated that the introduction of topo II-mediated DNA cleavages is prevented by intercalating agents such as ethidium bromide (20) and aclacinomycin (19). By use of an assay using UV cross-linking of topo II to DNA, we have demonstrated previously that this effect is mediated by an inhibition of the initial noncovalent DNA binding reaction of the enzyme (15). Similar to aclacinomycin, chloroquine inhibits the etoposide-mediated cytotoxicity and the formation of DNA breaks in cells (Fig. 4), and chloroquine also inhibits topo II-mediated DNA cleavage in vitro (Fig. 5). Accordingly, our data are consistent with the notion that chloroquine as aclacinomycin interferes with topo II-mediated DNA cleavage by inhibiting the enzyme from binding its DNA substrate. This effect abolishes topo II-mediated DNA cleavage and explains the antagonistic effect of chloroquine on the cytotoxicity of cleavage stimulators as etoposide, teniposide, m-AMSA, and daunorubicin.

It is interesting that the antimalarial agent chloroquine has such an impact on mammalian topo II. It is an open question whether chloroquine has the same effect on malarial topo II and whether this target is involved in the effect of chloroquine on the parasite. Certainly, many antimalarial agents are DNA binders (23). However, recent evidence strongly suggests that chloroquine affects the malaria parasite specifically by blocking a haem detoxification enzyme that polymerizes and detoxifies the haem moiety ferriprotoporphyrin IX in the acidic food vacuole (24) where the drug is accumulated due to base trapping (25).

An efficient in vivo protection of cells in neutral environments will enable a significant etoposide dose escalation, ultimately leading to an effective targeting of topo II in otherwise resistant acidic tumors. Recently, we demonstrated that the cation chelating bisdioxopiperazine derivative, ICRF-187, also abolishes DNA breaks and cytotoxicity of the topo II-targeting agents etoposide and daunorubicin (26). Although ICRF-187 is not trapped at acidic pHd, this drug is also a promising lead for the chemical synthesis of new proton trapped antagonists by virtue of its effective in vivo protection. Thus, we have shown that ICRF-187 in nontoxic doses provides full protection in vivo in healthy mice exposed to an etoposide dose killing 90% of the unprotected mice.4 One obvious goal is, therefore, the development of weak base ICRF-187 analogues.

In conclusion, by use of the antimalarial drug chloroquine, we have succeeded in obtaining a differential cytotoxicity of an established document.
anticancer agent, etoposide, entailing activity in an acidic environment comparable to that of most solid tumors with a lack of toxicity in cells with normal extracellular pH. This exploitation of extracellular antagonist trapping by acidic pH is a novel approach for regulation of anticancer drug effects.

ACKNOWLEDGMENTS

We thank Annette Nielsen for technical assistance.

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