Inhibitors of Topoisomerase II as pH-dependent Modulators of Etoposide-mediated Cytotoxicity

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ABSTRACT
Chloroquine intercalates into DNA and protects cells against topoisomerase II (topo II) poisons such as etoposide by hindering the DNA cleavage reaction of this target enzyme. Chloroquine, in contrast to etoposide, is a weak base and therefore barely enters the cell when the extracellular fluid is acidic, as is the case in most solid tumors. Such a pH-dependent drug interaction could be useful in targeting the cytotoxicity of topo II poisons toward solid tumors. Unfortunately, antagonistic chloroquine concentrations cannot be reached in vivo because of its unacceptable toxicity. Thus, antagonists with a higher therapeutic index are needed. We report here on the structure-activity relationship of several chloroquine and acridine analogues in a clonogenic assay. There were major differences in the cytotoxicity of the different compounds, with acridines being 50-fold more toxic than the chloroquine analogues. Several compounds were, however, able to antagonize etoposide-mediated cytotoxicity in a pH-dependent manner as chloroquine. Dependency on pH was lost if the aminoalkyl side arm of chloroquine was removed or lengthened by one CH₂ whereas pH dependency was strong with hydroxychloroquine. In contrast, the aminoalkyl side arm was clearly dispensable in the acridines because both quinacrine and 9-aminoacridine demonstrated profound pH dependency. The results from clonogenic assay were compared with cellular transport measurements and topo II enzyme inhibition. Compounds with the most marked pH-dependent intracellular accumulation were also the best pH-dependent protectors of etoposide cytotoxicity, clearly supporting the hypothesis that extracellular pH can be used to regulate topo II poisoning.

INTRODUCTION
The catalytic cell cycle of the nuclear enzyme topo II is the target of some of the most used antitumor agents, e.g., the epipodophyllotoxins etoposide and teniposide (1). A problem with these drugs is their low therapeutic index. Obviously, a more successful therapy would be possible if cytotoxicity could be targeted specifically to tumor cells. However, tumor-specific alterations are disappointingly few. One physiological difference between normal cells and tumor cells is their extracellular microenvironment, where solid tumors tend to develop a pH_e, which is about 0.5 pH units lower than in normal tissues (2–4). This pH difference could thus be suitable for targeting, as suggested by Newell et al. (3).

Etoposide-induced cell kill can be antagonized by distinct drug types. It has been demonstrated that the intercalating drugs aclorubicin, chloroquine, and the cardioprotecting agent ICRF-187 (dextrazoxane) antagonize the cytotoxicity of etoposide in vitro and in vivo (5–8). These so-called catalytic inhibitors appear to act in a different manner on the catalytic cycle of topo II than the classical poisons such as etoposide, teniposide, and many of the anthracyclines. They inhibit the enzyme without stabilizing the cleavable DNA-enzyme complex formation, thus being able to antagonize the action of the topo II poisons (reviewed in Ref. 9). As aclorubicin, chloroquine also antagonizes both topo I-mediated DNA single-strand breaks and the cytotoxicity of camptothecin (10).

Chloroquine is a weak base; therefore, it is ionized at low pH and consequently is unable to transverse the plasma membrane. The antagonistic effect on both etoposide and camptothecin cytotoxicity is pH dependent in clonogenic assays. As we have shown previously, chloroquine accumulation is decreased 5-fold in cells when the pH_e is lowered from values that are neutral (pH = 7.4) to acidic (pH = 6.0). No protection against etoposide by chloroquine is observed at pH_e = 6.5, whereas at pH_e = 7.4, etoposide cytotoxicity is almost completely antagonized (7). Unfortunately, in contrast to aclorubicin (11), chloroquine in itself is too toxic to protect against etoposide toxicity in mice.4

To study structure-activity relationships and develop less toxic antagonists, we have investigated a number of quinoline derivatives and acridine analogues to identify critical structures for the pH-dependent antagonism of etoposide cytotoxicity with the goal of targeting acidic tumors.

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3 The abbreviations used are: topo, topoisomerase; pH_e, extracellular pH; kDNA, kinetochore DNA; MTD, maximal tolerated dose; GS1, 7-chloro-4-(3-hydroxy-propylamino)quinoline; GS2, 7-chloro-4-(5-diethylamino-1-methylpentylamino)quinoline; GS3, 7-chloro-4-(3-diethylamino-1-methylpropylamino)quinoline; GS4, 7-chloro-4-((N’2-DEAE))-N-methylhydrazino)quinoline.
4 Unpublished observations.
MATERIALS AND METHODS

Cell Lines

Human small cell lung cancer OC-NYH (GLC-2; Ref. 12) and NCI-H69 (13) were grown in RPMI 1640 supplemented with 10% FCS plus penicillin and streptomycin.

Drugs/Chemicals

The chemical structures of the quinolines and acridines are shown in Fig. 1. All purchased and synthesized drugs were of at least 97% purity. Amodiaquine [7-chloro-4-(3-diethyl-amino-methyl-4-hydroxy-anilino)quinoline]; primaquine [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline, 2H3 PO 4 ]; quinine [α-(hydroxy-(6-methoxy-quinolinyl-4)methyl)-3-vinylquinuclidine]; quinacrine [mepacrine; 3-chloro-9-(4-diethyl-amino-1-methylbutylamino)-7-methoxyacridine, 2HCl]; and chloroquine [7-chloro-4(4-diethylamino-1-methylbutylamino)-7-methoxyacridine, 2HCl]; and hydroxychloroquine [7-chloro-4(4-(ethyl-(2-hydroxyethyl)amino-1-methylbutylamino)quinoline, H2 SO 4 ] were obtained from Sigma Chemical Co. (St. Louis, MO). 9-Aminoacridine, HCl, H2 O was purchased from Aldrich (Steinheim, Germany). Hydroxychloroquine [7-chloro-4(4-(ethyl-2-hydroxyethyl)amino-1-methylbutylamino)quinoline, H2SO4] was obtained from Erco- pharm (Kvistgaard, Denmark). [14C]Chloroquine was obtained from Amersham (Buckinghamshire, United Kingdom). Aclarubicin was purchased from Lundbeck (Copenhagen, Denmark), and ICRF-187 was from Chiron (Amsterdam, the Netherlands).

The following compounds were synthesized de novo, and the results were checked by fast atom proton bombardment mass spectroscopy and elementary analysis:

GS1. A mixture of 3-hydroxypropylamine (0.75 g; 10 mmol), 4,7-dichloroquinoline (1.98 g; 10 mmol) and phenol (0.94 g; 10 mmol) was heated with stirring at 140°C for 5 h. The reaction mixture was cooled to 100°C, poured into water, and stirred to induce crystallization. It was then washed with water and filtered, and the solid was recrystallized several times from 96% ethanol to give 1.79 g (76%) and finally dissolved in water.

GS2. A mixture of 4-diethylamino-2-aminohexane (0.4 g; 2.3 mmol), 4,7-dichloroquinoline (0.46 g; 2.3 mmol), and phenol (0.22 g; 2.3 mmol) was heated at 160°C for 6 h. The reaction mixture was cooled and poured into 30% aqueous sodium hydroxide and stirred for 1 h. The aqueous solution was extracted with ether, and the ether extracts were washed with water and dried over sodium sulfate. Evaporation of the solvent gave an oil that was distilled at 160–180°C and 0.06 mm Hg. The oil solidified on standing, and the product was recrystallized from hexane:benzene 1:1 to give 0.44 g (57%) and then dissolved in water.

GS3. A mixture of 4-diethylamino-2-aminobutane (0.5 g; 3.5 mmol), 4,7-dichloroquinoline (0.63 g; 3.5 mmol), and phenol (0.33 g; 3.5 mmol) was heated at 160°C for 6 h. The reaction mixture was cooled and poured into 30% aqueous sodium hydroxide and stirred for 1 h. The aqueous solution was extracted with ether, and the ether extracts were washed with water.

Fig. 1 Chemical structure of the chloroquine and acridine analogues. Arrows and figures refer to the “Discussion” section.
and dried over sodium sulfate. Evaporation of the solvent gave an oil that was distilled at 150–180°C and 0.07 mm Hg. The oil solidified on standing, and the product was recrystallized from hexane:benzene 1:1 to give 0.66 g (62%) and dissolved in DMSO.

**GS4.** A mixture of N-(2-DEAE)-N-methylhydrazine (1.00 g; 6.6 mmol), 4,7-dichloroquinoline (1.19 g; 6.0 mmol), and phenol (2.8 g; 30 mmol) was heated with stirring at 150°C for 7 h. The reaction mixture was cooled, poured into 10% aqueous KOH, and extracted with dichloromethane. The organic extract was washed with water and dried over sodium sulfate. Evaporation of the solvent gave an oil that was distilled at 140–150°C and 0.07 mm Hg. The oil solidified on standing, and the product was recrystallized from hexane to give 0.59 g (32%) and finally dissolved in DMSO.

**Clonogenic Assay**

Drug cytotoxicity was assessed by colony formation in soft agar with a feeder layer containing sheep RBCs as described previously (14). Single-cell suspensions in RPMI 1640 supplemented with 10% FCS (complete medium) were exposed for 1 h to the drugs at 37°C at the desired pH and concentrations and washed twice with complete medium at 37°C at pH 7.4. The pH was adjusted with 5 M HCl. Approximately $2 \times 10^6$ cells were plated to obtain 3000–4000 colonies in the control dishes. The colonies were counted after 3 weeks incubation, and survival was calculated as compared with control cells. All experiments were done in triplicate.

**Drug Accumulation**

Two million cells in single-cell suspension in 2 ml of PBS containing dialyzed 5% FCS were incubated for 30 min with DNase I, type II (Sigma D4527). Hereafter, we essentially followed the procedures described by Skovsgaard (15). Twenty μl of the drug were added to produce the following final concentrations: 350 μg/ml primaquine, 20 μg/ml quinacrine, 10 μg/ml amodiaquine, and 5 μg/ml 9-aminoacridine. Cells were then pelleted at 3000 × g for 2 min at 4°C and washed twice with 7 ml of ice-cold Ringer’s solution at 4°C to remove the extracellular drug. The drug then was extracted from the cells with 3 ml of 0.3 M HCl in 50% ethanol.

The total fluorescence of the supernatant solution after centrifugation at 3000 × g for 10 min was determined in duplicate by spectrofluorophotometry (9-aminoacridine excitation and emission wavelengths 373 nm and 456 nm; quinacrine 438 nm and 456 nm, respectively). Spectrophotometric measurements of extracts of primaquine and amodiaquine were done in triplicate at wavelengths of 335 nm and 456 nm, respectively.

Accumulation of [14C]chloroquine was assessed by liquid scintillation counting as described previously (7).

**Decatenation Assay**

topo II catalytic activity was measured by kDNA decatenation. $\text{[3H]klDNA}$ was isolated from *Crithidia fasciculata* (American Type Culture Collection, Manassas, VA) as described by Sahai and Kaplan (16). Briefly, purified topo IIa was incubated with 0.2 mg of kDNA for 15 min at 37°C in a final volume of 20 ml in a buffer containing [50 mM Tris-Cl (pH 8), 120 KCl, 10 mM MgCl$_2$, 1.0 mM ATP, 0.5 mM DTT, and 30 mg of BSA/ml]. After addition of stop buffer/loading dye mix (5% Sarkosyl, 0.0025% bromphenol blue, and 25% glycerol), samples were loaded on 1% agarose/0.5% ethidium bromide gels and run in TBE buffer containing 0.5 mg/ml ethidium bromide at 100 V for ~50 min and photographed under UV light. In addition, loading wells were cut out and scintillation counted to obtain numerical values.

**RESULTS**

The structure-related strategy used in testing the different compounds is outlined in Fig. 1.

**Dose-finding Studies.** Initially, the MTD of the modulator drugs were determined in a series of clonogenic assays (not shown). MTD was defined as the highest achievable dose in the pH range of 6.3–7.4, resulting in less than LD$_{20}$. These concentrations (Table 1) were the ones subsequently used in the clonogenic assays, both alone and in combination with 20 μM etoposide.

Amodiaquine, GS2, and the neutral compound 4,7-dichloroquinoline, were far more potent (MTD, 10–50 μM) than the rest of the 4-aminoquinolines (MTD, 300–550 μM). The acri-dines 9-aminoacridine and quinacrine also were potent cytotoxic compounds, with MTDs of 15 and 20 μM, respectively.

**The pH-dependent Cytotoxicity and Antagonism of Etoposide.** We then performed a series of clonogenic assays to determine the pH-dependent cytotoxicity of etoposide alone and in combination with the various drugs, as depicted in Fig. 2. As shown previously, etoposide was more toxic at neutral than at acidic extracellular values. It appears that the cytotoxicity of etoposide is somewhat variable from experiment to experiment, most likely attributable to the fact that the experiments were conducted over a period of several months. For the experiments to be suitable for direct comparison, the required etoposide cytotoxicity had to exceed 90% at all pH levels. The ability of the drugs to antagonize 20 μM etoposide is illustrated in Fig. 3, showing the fold protection, which was calculated as the relative survival of colonies treated with the combination of modulator drug and etoposide to the survival when treated with etoposide alone at neutral (pH 7.3–7.5) as well as acidic pH$_1$ (pH 6.3–6.5). The minor change of the chloroquine alkyl side chain resulting in hydroxychloroquine (step 2 in Fig. 1) as well as the change of the ring structure (step 8, quinacrine) did not have any signifi-

<table>
<thead>
<tr>
<th>Drug</th>
<th>MTD (μM)</th>
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<tr>
<td>Quinine</td>
<td>650</td>
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<tr>
<td>Hydroxychloroquine</td>
<td>550</td>
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<tr>
<td>Primaquine</td>
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<td>Chloroquine</td>
<td>500</td>
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<td>GS3</td>
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<td>GS4</td>
<td>300</td>
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<td>GS1</td>
<td>50</td>
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<tr>
<td>4,7-Dichloroquinoline</td>
<td>25</td>
</tr>
<tr>
<td>GS2</td>
<td>20</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>15</td>
</tr>
<tr>
<td>9-Aminoacridine</td>
<td>10</td>
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Table 1 MTDs of the drugs used in the clonogenic assay
Fig. 2 Results of the clonogenic assays. Bars, SE of triplicate platings.
Fig. 2  Continued.
cant impact on the marked ability to antagonize the cytotoxicity of etoposide at neutral pH in vitro. Removal of the side chain from the acridine ring system (step 9, 9-aminoacridine) did not influence the antagonism or pH dependency. At neutral pH, the fold protection for these three compounds were in the range of 80–430, declining to 1–4 at acidic pH.

The insertion of a ring structure instead of an alkyl side chain at the four positioned substituent in the quinoline ring (steps 7 and 11; amodiaquine and quinine, respectively) had a pronounced effect on antagonism. Although a weak antagonist, the cytotoxicity of amodiaquine in combination with etoposide was pH dependent as opposed to primaquine (step 10), which was in accordance with the measurements of intracellular drug accumulation (Fig. 4). The most pronounced structure-activity relationship was seen in step 4; the elongation of the alkyl side chain by only one CH2 in GS2 resulted in complete loss of etoposide antagonism and far greater toxicity as compared with the rest of the 4-aminoquinolines, apart from 4,7-dichloroquinolone.

Fig. 3 The fold protection of the different compounds in acidic (the left of each pair of columns) and neutral (right columns) pH. The fold protection expresses the degree of protection conferred by the combination of the modulator compound +20 μM etoposide to the cytotoxicity of 20 μM etoposide alone.

Fig. 4 Relative intracellular drug accumulation as a function of pH. Ordinates, pH; abscissae, relative intracellular drug concentration. Bars, SE of two or three measurements.
line. This neutral compound likewise had only weak antagonistic properties with relatively small pH variation (step 1).

Shortening of the alkyl side chain, on the other hand, had no effect at all (GS4, step 6) or a slightly diminishing influence on the antagonism (GS1 and GS3, steps 5 and 3, respectively). The potency of these drugs was comparable.

To rule out the possibility that our findings were cell line specific, we also conducted a small series of clonogenic assays with the more commonly used NCI-H69. These showed that both chloroquine and 9-aminoacridine, but not primaquine or GS2, protected against etoposide toxicity at neutral pH (not shown).

**Antagonism and pH-dependent Cytotoxicity at Low Dose.** We repeated the clonogenic assays at pH 7.4 and 6.7 in two doses, i.e., MTD and one-fifth of the MTD. In this way, we were able determine whether the etoposide antagonism and/or pH-dependent cytotoxicity was dose dependent as well. Fig. 5a shows the results from the assay using chloroquine as the test drug. The pH dependency and the antagonism of etoposide cytotoxicity are clearly reduced at one-fifth of the MTD. Dose-dependent reduction of etoposide antagonism and pH dependency were features of all of the antagonists apart from quinacrine (Fig. 5b) and 9-aminoacridine. These two acridines exhibited totally unchanged antagonism as well as pH dependency at the low concentrations.

**Drug Accumulation.** Next, measurements of intracellular drug accumulation as a function of extracellular pH were performed with chloroquine, primaquine, amodiaquine, quinacrine, and 9-aminoacridine. The intracellular concentrations at neutral pH were highest in all experiments; the results are presented as the relative values compared with neutral values (Fig. 4). The accumulation of primaquine was almost independent of extracellular acidity, whereas lowering of the pH₄ had an obvious inhibitory effect on cellular uptake of the other drugs.
tested. In particular, the effect was pronounced on 9-aminoacridine and quinacrine, with a 75 and 50% reduction at pH 6.9 and 6.7, respectively.

Effect of the Test Drugs on topo II-mediated kDNA Decatenation. Finally, the relative inhibition of the test drugs on topo II-mediated decatenation of the kDNA network at the three dose levels is shown in Fig. 6. Chloroquine, OH-chloroquine, GS3, 9-aminoacridine, and quinacrine exhibited dose-dependent inhibition of the topo II enzyme activity, whereas amodiaquine, and GS2 did not. Primaquine and 4,7-dichloroquinoline were only tested at two dose levels because of a lack of solubility but appeared not to affect the decatenation at these concentrations. GS1 was only a weak inhibitor.

DISCUSSION

We have shown previously that the antimalarial chloroquine antagonizes etoposide-mediated cytotoxicity in a dose-dependent manner (7). Furthermore, the antagonism is almost complete at pH 7.4, whereas the protection is abolished at pH 6.5. It would therefore be expected that other quinoline compounds could have the same effect.

Despite minor changes in the alkyl side chain of the chloroquine molecule, i.e., shortening and hydroxylation as described, the 4-aminoquinolines retain their ability to protect cells against etoposide-mediated cytotoxicity in a pH-dependent manner in vitro. However, major changes, i.e., insertion of a ring structure in the side chain (amodiaquine) or lengthening or even removal of the alkyl side chain of chloroquine (GS2 and 4,7-dichloroquinoline, respectively), markedly reduced etoposide antagonism. The cytotoxicity of these drugs is enhanced as well. The decatenation assay measures the direct effect of the test drugs on the catalytic cycle of topo II. Even at high concentrations, amodiaquine and GS2 do not inhibit the topo II-mediated decatenation of kDNA. In contrast, 9-aminoacridine and quinacrine as well as the chloroquine analogues exhibit dose-dependent inhibition. Thus, the lack of antagonism of GS2 in the clonogenic assays could be explained by low drug uptake. Amodiaquine, which displays weak antagonistic properties in the clonogenic assays at neutral pH, presumably exerts its effect by another mechanism other than interference with the DNA-topo II complex.

Compared with the majority of the 4-aminoquinolines, the acridine-based compounds (quinacrine and 9-aminoacridine) are cytotoxic at very low concentrations. Interestingly, the pronounced etoposide antagonism exhibited by both drugs does not seem to relate to an alkyl side chain as seen with the tested 4-aminoquinolines. The results from the clonogenic assays can be related to the measurements of intracellular drug accumulation and thereby confirm the role of the cell membrane in the regulation of the pH-dependent antagonism of etoposide antagonism. For example, the pH-dependent drug transport profile is most marked for 9-aminoacridine. At low pH, the measured intracellular concentration is indeed minimal. However, the clonogenic assays measuring dose-relationship showed completely unchanged pH dependency and etoposide antagonism for 9-aminoacridine and quinacrine, even at doses as low as one-fifth of the MTD, in contrast to the dose-dependent antagonism of chloroquine. Therefore, the combined effects of the accumulation and drug potency profile conveniently link the results of the transport measurements and the clonogenic assays.

The clinical usefulness of cytotoxic drugs is limited by development of drug resistance and by unwanted side effects. Both problems are at least somewhat (and inversely) related to dose levels. Accordingly, it would be desirable to be able to target cytotoxicity drugs directly to the malignant tissue, thus reducing systemic side effects, and furthermore attempt to in-
crease their doses to overcome drug resistance. As opposed to chloroquine and hydroxychloroquine, the pH-dependent etoposide antagonism of the acridines, i.e., quinacrine and 9-aminoacridine, was present at low concentrations. This might be beneficial in a toxicological sense, so that the potential of using these drugs clinically as an instrument for targeted chemotherapy could be studied in preclinical animal tests. Hence, these in vitro results do encourage further in vivo studies of these two drugs in particular. In contrast, the de novo synthesized drugs, i.e., GS1, GS2, GS3, and GS4 do not seem to be improved agents.

Other catalytic inhibitors of topo II can protect against etoposide cytotoxicity in mice. Thus, aclarubicin is an effective protector (11) that intercalates in the same manner as chloroquine, i.e., it interferes with the initial binding of topo to DNA (17, 18). However, this antagonism is not pH dependent. Furthermore, the bisdioxopiperazine ICRF-187 (dextraoxazone) is a very potent catalytic inhibitor in vivo. It exerts its effect much later in the catalytic cycle of topo II, i.e., by locking the topo II after religation (17, 19–22). However, ICRF-187 is not a weak base, and its interaction with etoposide and other topo II poisons is not dependent on pH. We believe that the possibility of using the naturally occurring differences in pH between malignant and nonmalignant cells deserves attention. By demonstrating that several quinolines and acridines can antagonize the topo II poison etoposide in a pH-dependent manner in vitro, the track has been laid for further preclinical animal testing of this hypothesis.

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