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pH-Dependent Regulation of Camptothecin-Induced Cytotoxicity and Cleavable Complex Formation by the Antimalarial Agent Chloroquine

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ABSTRACT. Two classes of drugs interact with DNA topoisomerase (topo) I, namely topoI poisons such as the camptothecins, which create DNA single-strand breaks and the catalytic inhibitors, which do not. Here, we demonstrate that the antimalarial agent chloroquine is a catalytic inhibitor of eukaryote topoI, as the drug inhibited topoI-mediated DNA relaxation. Chloroquine is known to be a topoII catalytic inhibitor and as such is able to inhibit the activity of a topoII poison, i.e. etoposide. We now show that chloroquine also inhibits the topoI poison camptothecin as camptothecin-stimulated nicking of plasmid DNA was inhibited by chloroquine. These observations also apply to endogenous topoI in whole cells. Accordingly, camptothecin-induced single-strand breaks as well as cytotoxicity were antagonised by chloroquine. Further, in a band depletion assay in whole cells, chloroquine prevented camptothecin-mediated topoI trapping, indicating that chloroquine inhibits topoI by interfering with the DNA binding step of the enzyme. In contrast to camptothecin, chloroquine 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. This leads to the possibility of directing cytotoxicity to solid tumors with low extracellular pH by combining a neutral anticancer agent, i.e. camptothecin with a weak base antagonist, i.e. chloroquine. To test the feasibility of this principle, we investigated the drug combination at varying extracellular pH. We found that the antagonising effect of chloroquine on camptothecin-mediated trapping of topoI and DNA single-strand break formation was abolished at acidic extracellular pH. In a clonogenic assay, camptothecin in combination with chloroquine selectively killed cells at low pH (6.2), while camptothecin cytotoxicity was antagonised by chloroquine at normal pH (7.2). In conclusion, we show that the topoI catalytic inhibitor chloroquine inhibits camptothecin and that chloroquine can target the cytotoxic effect of camptothecin to tumor cells in acidic environments. BIOCHEM PHARMACOL **54**;3:373–380, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. chloroquine; camptothecin; DNA topoisomerase I; tumor pH; targeting of cytotoxicity

The camptothecins represent a promising class of anticancer agents with a novel mechanism of action. These drugs target the nuclear enzyme DNA topoisomerase I (topoI)§ [1]. The enzyme functions by introducing the concerted breakage and religation of single-stranded DNA, thereby releasing the torsional strain in the DNA as replication and transcription occur. Camptothecin (CPT) affects the rejoining step by stabilising the topoI-DNA intermediate known as the cleavable complex, leading to extensive accumulation of DNA single-strand breaks (reviewed in [2, 3]). CPT has been shown to be effective against several experimental tumors and a number of semisynthetic derivatives are currently in phase II trials [4].

Due to encouraging response rates, it is expected tha these compounds will become part of standard cance treatment in the future. Consequently, pharmacologica regulation of CPT is of major interest. Ultimately, such regulation could lead to more selective therapy by use of an antagonising drug in combination with CPT in order to protect normal tissue. This principle is currently being used in the clinic, e.g. folinic acid rescue of methotrexate toxicity and protection by MESNA against high-dose cyclophosphamide toxicity. Candidates for such pharmacological modulation may be sought among agents that modulate the catalytic action of topoI. TopoI active drugs can be categorised in two groups, i.e. cleavable complex forming drugs, known as topoI poisons (CPT), and drugs that act upon the enzyme without cleavable complex formation, so-called catalytic inhibitors such as the acridines, distamycin A and aclarubicin with strong affinity to the DNA substrate [5–7] and β-lapachone, which affects the enzyme itself [8]. Such regulation of CPT is only meaningful in the clinical setting if the modulating agent selectively antagonises the effect of CPT in normal tissues

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§ *Abbreviations:* TopoI and II, DNA topoisomerase I and II; FCS, fetal calf serum; NB, nucleus buffer; CPT, camptothecin; CLQ, chloroquine; SCLC, small cell lung cancer.

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without reducing the cytotoxic effect on tumor cells. In an effort to meet these requirements, we previously presented a model for the selective killing of tumor cells by the DNA topoisomerase II (topoII)-directed drug etoposide. The model exploits the fact that the pH of the extracellular fluid in solid tumors is lowered by 0.5 on average compared to normal tissue [9]. We showed that the intercalating antimalarial agent chloroquine (CLQ) protects against etoposide-induced cytotoxicity. Furthermore, protection by CLQ is dependent on extracellular pH as CLQ is trapped outside cells at low pH because of its weak base properties. The differential uptake of the antidote CLQ allows for full protection at normal pH without reducing etoposide cytotoxicity in an acidic environment comparable to that of most solid tumors [10].

In this study we report that CLQ inhibits the catalytic process of topoI *in vitro* as well as in whole cells, resulting in protection form CPT-induced cell death. By use of CLQ as a pH-dependent CPT antagonist, we show that CPT cytotoxicity can be targeted to tumor cells in acidic environments.

MATERIALS AND METHODS

Cell Lines

The human small cell lung cancer (SCLC) cell line OC-NYH (also designated GLC-2) was used [11]. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) plus penicillin and streptomycin. All experiments were carried out on cells in exponential growth.

Drugs

Drugs used in the clonogenic assay were dissolved immediately before use and diluted more than 100-fold with tissue culture medium. CLQ and CPT (both Sigma Chemical Co., St. Louis, MO, USA) were dissolved in water and in dimethyl sulfoxide, respectively. In the alkaline elution assay, aliquots of 10 mM kept at −80 °C were thawed just prior to use. Radiolabelled [^{3}H]-CPT (27 Ci/mmol) was purchased from Moravek Biochemicals Inc., Brea, CA, USA.

Clonogenic Assay

Cytotoxicity was assessed by colony formation in soft agar with a feeder layer containing sheep red blood cells as described previously [12]. Experiments with fixed pH at 7.4 (Fig. 5) were performed as follows. 2×10^4 cells were pre-treated with or without CLQ for 20 min in RPMI medium supplemented with 10% FCS plus penicillin and streptomycin followed by an additional 24 hr incubation with CPT added to the culture medium. Cells were washed twice before plating as single-cell suspensions. After 14–21 days, the colonies were counted and survival was calculated as compared to control cells. The number of colonies obtained in controls was approximately 5000. Experiments with varying pH (Fig. 8) were performed as above except that after 60 min CLQ pre-treatment at varying pH, medium was removed and cells were incubated with CPT for 24 hr at pH 7.4, as OC-NYH cells are not adapted to prolonged incubation at acidic pH.

TopoI-mediated Relaxation of Supercoiled Plasmid DNA

Reaction mixtures consisted of 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine, 0.01% (w/v) BSA, 500 ng pBR322 DNA (Promega Co., Madison, WI, USA), 0.02 ng of topoI, isolated from S. cerevisiae (a generous gift from Ole Westergaard, Department of Molecular and Structural Biology, Aarhus University, Aarhus, Denmark) and the indicated concentrations of CLQ. Final volume was 20 μL. Incubation was done at 37 °C for 30 min. The reaction was stopped by a 60 min incubation with 0.5% (w/v) SDS and 200 ng/mL proteinase K (final concentration). Samples were run on a 1% agarose gel. After electrophoresis, gels were stained with ethidium bromide and photographed under UV light. To distinguish the supercoiling effect of CLQ on relaxed DNA from unreacted supercoiled substrate DNA due to inhibition of topoI, we included samples with CLQ added to relaxed DNA after proteinase K digestion of topoI was completed.

TopoI-induced Cleavage of Plasmid DNA

Nicking of plasmid DNA by CPT in the presence of topoI was performed as described by Hsiang [13] with minor modifications. The reaction mixture contained 35 mM Tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine, 0.01% (w/v) BSA and 500 ng of pBR322 DNA in a final volume of 20 μL. CLQ pre-incubation was done for 15 min followed by addition of 12 ng topoI and CPT in a total volume of 20 μL. After 30 min at 37 °C the reaction was terminated by the addition of SDS and proteinase K to final concentrations of 0.5% (w/v) and 200 ng/mL, respectively. Digestion of protein was performed for 60 min at 37 °C. After addition of loading buffer, samples were run on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. The inclusion of ethidium bromide in the gel enables the separation of nicked DNA from supercoiled and relaxed DNA. Nicked DNA migrates to a position at the top of the gel. In contrast, supercoiled and relaxed DNA co-migrate to a position at the bottom of the gel. Gels were photographed under UV light.

Measurement of DNA Single-strand Breaks

DNA damage was quantitated by the alkaline elution filter method as described by Kohn [14]. [^{3}H]-thymidine-labelled L1210 cells used as internal standard were exposed to 100 μM H_2O_2 for 60 min on ice, corresponding to an irradiation dose of 300 rad [15]. [^{14}C]-thymidine-labelled

cells were incubated with or without CLQ in medium for 10 min at varying pH followed by an additional 50 min of CPT exposure at 37 °C. Mixing of standard and experimental cells was carried out immediately prior to lysis. DNA was eluted at pH 12.1 under deproteinising conditions using a 2.0 μM pore-size Nucleopore filter (Corning Costar Co., Cambridge, MA, USA). Fractions were collected at 20 min intervals for 2 hr at an elution rate of 0.125 mL/min.

Band Depletion Assay

Cells were pre-treated in medium with or without CLQ at varying pH for 15 min followed by exposure to CPT for an additional 30 min at 37 °C. Subsequently, crude nuclear extracts were prepared as previously described, with minor modifications [16]. All steps were performed at 4 °C. Cells were washed twice in a hypotonic nucleus buffer (NB) [2 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 0.2 mM dithiothreitol, 1 mM phenyl-methyl-sulfonyl-fluoride, pH 6.5], and subsequently lysed for 5 min in NB supplemented with 0.3% (v/v) Triton X. After washing with NB, proteins were extracted from nuclei in NB with 350 mM NaCl for 30 min. Insoluble nuclear fragments were spun down at 15,000 × g and the supernatants were collected. Equal amounts of protein measured by the Bradford protein assay [17] were loaded on an SDS-PAGE gel. Proteins were blotted and detected by use of an affinity-purified serum from scleroderma patients containing antibodies against topoI (TopoGEN, Columbus, OH, USA) overnight at 4 °C. Alkaline phosphatase-conjugated rabbit antihuman antibody (Dako, Copenhagen, Denmark) was used as secondary antibody. Quantitation of immunoreactive bands was done by densitometric scanning.

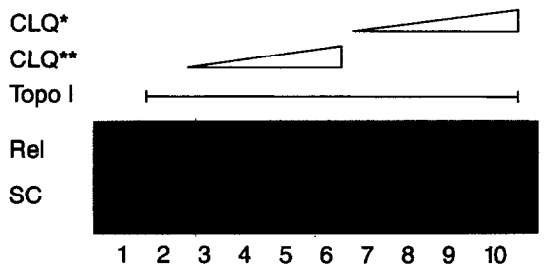

FIG. 1. Chloroquine inhibition of topoisomerase I-mediated relaxation of supercoiled DNA. pBR 322 DNA was reacted with 0.02 ng topoI in the presence of chloroquine (CLQ). Drug was added to substrate DNA immediately before topoI, indicated by CLQ (lanes 3–6) or to relaxed DNA after proteinase K digestion of topoI was completed, indicated by CLQ* (lanes 7–10). DNA separation was done on a 1% agarose gel, subsequently stained with ethidium bromide. Lane 1, no topoI; lane 2, topoI, no drug; lanes 3–6, topoI, CLQ at 0.1, 0.3, 1 and 3 mM; lanes 7–10, topoI, CLQ added after proteinase K digestion at 0.1, 0.3, 1 and 3 mM. Rel and SC indicate position of relaxed and supercoiled DNA, respectively.**

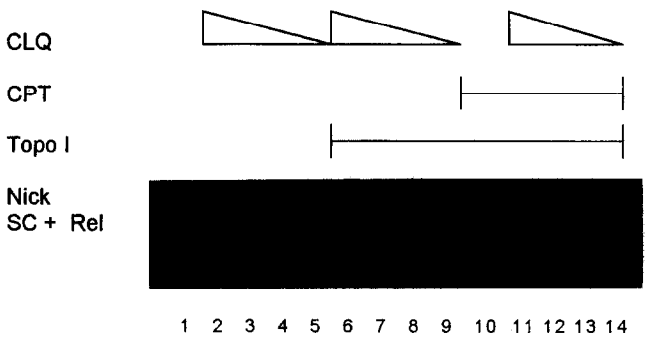

FIG. 2. Protection of camptothecin induced nicks in plasmid DNA pBR 322. DNA was reacted with 12 ng topoI in the presence of the indicated drugs. Chloroquine (CLQ) was added 15 min before topoI and camptothecin (CPT). DNA was separated on a 1% agarose gel containing ethidium bromide. Lanes 1–5, no topoI; lane 1, plasmid control; lane 2, 3 mM CLQ; lane 3, 1 mM CLQ; lane 4, 0.3 mM CLQ; lane 5, 0.1 mM CLQ; lanes 6–9, as lanes 2–5 with topoI included; lane 10, topoI and 1 μM CPT; lanes 11–14 as lanes 2–5 with topoI and 1 μM CPT included. Positions of nicks as well as supercoiled (SC) and relaxed (Rel) DNA are indicated on the left.

Accumulation of [^{3}H]-camptothecin

5 × 10^6 OC-NYH cells in single cell suspensions were incubated with DNase I (Sigma Chemical Co.) at 0.025% for 30 min to disintegrate nuclei from dead cells [18]. Thereafter, cells were incubated with or without 2 mM CLQ followed immediately by addition of [^{3}H]-CPT at 5 μM in PBS (57.0 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 51.0 mM Na_2HPO_4, 9.0 mM NaH_2PO_4, pH 7.45) to which 5% FCS and 10 mM glucose were added. pH was adjusted by addition of HCl. After 60 min at 37 °C, the cells were spun down at 150 g for 5 min and washed twice with ice-cold PBS. Cell pellets were solubilised in 0.8 mL 0.5 N KOH at 70 °C for 1 hr and analysed in a Packard scintillation counter (Canberra Packard International, Zürich, Switzerland) [19].

RESULTS

The effect of CLQ on topoI-mediated relaxation was examined. Fig. 1 shows that CLQ is a catalytic inhibitor of topoI. At 3 mM CLQ inhibition appears complete with a prominent band corresponding to supercoiled DNA migrating faster than the relaxed form (lane 6). Due to the DNA unwinding nature of CLQ, one could argue that the DNA merely migrates faster because of the introduction of positive superturns by CLQ. To rule out this possibility, we incubated relaxed DNA with CLQ at the same concentrations. As seen in Fig. 1, the increased mobility is not merely caused by CLQ-mediated supercoiling of relaxed DNA but can only be accounted for as a result of inhibition of topoI relaxation by CLQ (compare lane 6 with lane 10).

In contrast to CPT, CLQ does not stimulate cleavable complex formation. In fact, CLQ inhibits CPT-stimulated cleavable complexes in plasmid BR322 DNA. As shown in Fig. 2, CLQ reduced the amount of nicked DNA mediated

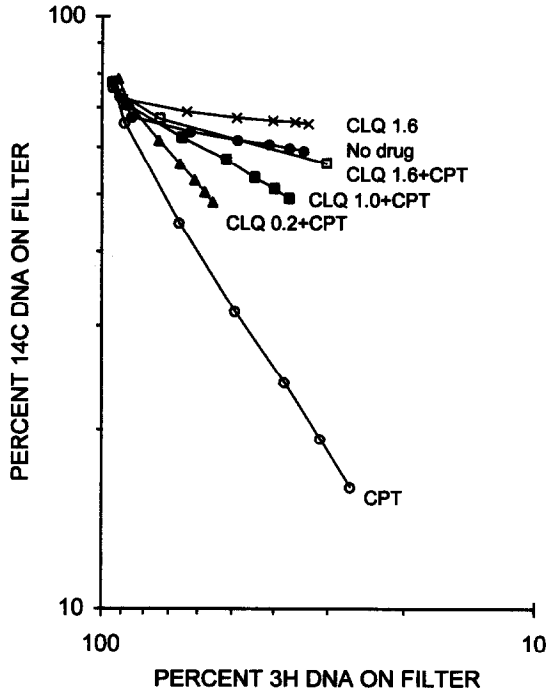

FIG. 3. Protection of camptothecin-induced DNA-single-strand breaks by chloroquine. DNA single-strand breaks were measured by alkaline elution. [^{14}C]-thymidine-labelled OC-NYH cells were exposed to 0.2 (triangle), 1.0 (filled square) and 1.6 mM (open square) chloroquine (CLQ) or to no CLQ (open circle) at pH 7.4 for 10 min followed by an additional 50 min camptothecin (CPT) treatment at 0.5 μM at 37 °C. Control cells were exposed to no drug (filled circle) or to CLQ only (cross). Internal standard L1210 cells were labelled with [^{3}H]-thymidine. Percent of [^{14}C] and [^{3}H] labelled DNA remaining on filters are plotted logarithmically on the y- and x-axis, respectively. Numbers at each curve indicate concentration of CLQ in mM.

by CPT (lanes 11–14) compared to CPT alone (lane 10). In accordance with the relaxation assay (Fig. 1, lane 6), 3 mM CLQ antagonised CPT-mediated nicks. CLQ by itself (lanes 2–5) or mediated by topoI (lanes 6–9) did not induce nicking of DNA that exceeded background levels with supercoiled DNA only (lane 1). Cleavable complexes stimulated by a wide range of CPT concentrations were inhibited by CLQ. Using at least 10-fold higher concentrations of CPT resulted in the same degree of inhibition by CLQ (not shown).

In order to investigate if our observations on plasmid DNA translate to whole cells, we measured CPT mediated DNA single-strand breaks by the alkaline elution technique. The use of alkaline elution offers the advantage of an endogenous topoI acting on an intact chromatin structure. Indeed, we found that CLQ protects against CPT-mediated DNA breaks in a dose-dependent manner at pH 7.4 (Fig. 3). CLQ alone did not induce any single-strand breaks.

Inhibition of relaxation and CPT-induced strand breaks may be due to interference with topoI binding to its DNA substrate. To test this, we performed a band depletion assay measuring trapping of topoI to DNA in whole cells. CPT alone trapped topoI onto DNA as shown by the 80% reduction in high salt extractable topoI (Fig. 4, lane 2). In contrast, etoposide did not deplete extractable topoI (not shown). However, preincubation with 2 to 20 mM CLQ completely prevented nuclear topoI trapping (Figs. 4 and 7, lane 3). No change in extractable topoI was found in cells incubated with CLQ alone (Fig. 4, lane 4).

As cleavable complex formation is a prerequisite for CPT-induced cell death, one would expect CLQ to be capable of protecting against CPT-induced cell death. As seen in Fig. 5, an increasing fraction of CPT-treated cells survive with increasing CLQ concentration at pH 7.4. In fact, the survival fraction of cells treated with both CPT and CLQ at 290 μM equals that of cells treated with CLQ alone, corresponding to a protection against CPT of more than 2 logs or 6 to 7 cell doublings.

Previously, we demonstrated that CLQ, due to its weak base properties, is trapped outside cells at a low extracellular pH [10], comparable to that found in most solid tumors. Consequently, we tested if CLQ protection measured by alkaline elution and band depletion assay would be abolished at low extracellular pH. Indeed, CLQ protection was very sensitive to variations in pH. As shown in Fig. 6, CLQ protection against CPT-mediated DNA-single-strand formation was abolished when the pH was reduced from 7.4 to 7.0. In accordance with this, CLQ did not prevent CPT-

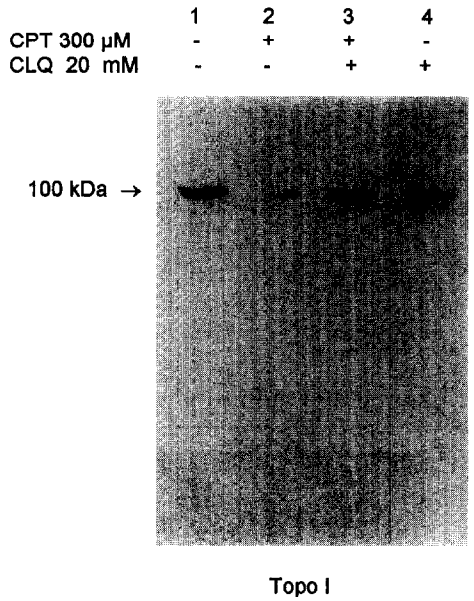

FIG. 4. Prevention of camptothecin-mediated trapping of topoisomerase I to DNA by chloroquine in whole cells. TopoI trapping was assessed by a band depletion assay. At pH 7.4, OC-NYH cells were incubated at 37 °C with (lanes 3 and 4) or without (lanes 1 and 2) chloroquine (CLQ) at 20 mM for 15 min followed by an additional 30 min of dimethyl sulfoxide (lanes 1 and 4) or camptothecin (CPT) incubation at 300 μM (lanes 2 and 3). 350 mM NaCl nuclear extracts were prepared and topoI was detected by Western blotting. Samples were run on the same gel and the lanes depicted were subsequently juxtaposed. Arrow indicates the position of the 100 kDa molecular weight marker.

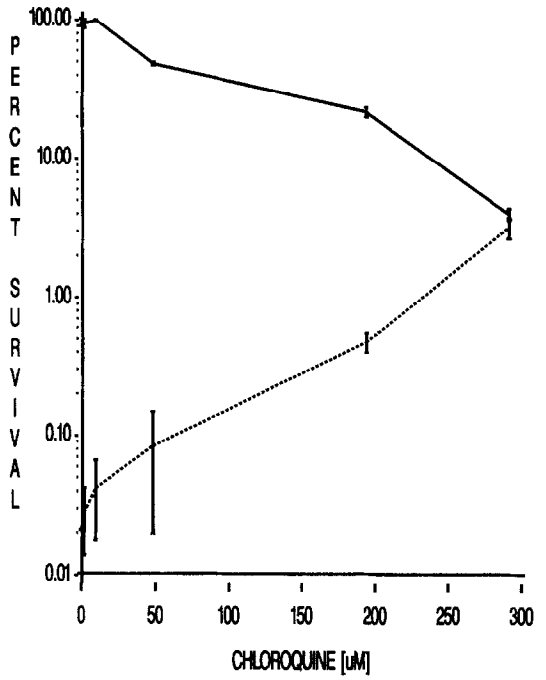

FIG. 5. Antagonism of camptothecin-induced cytotoxicity by chloroquine. Cytotoxicity was assessed by the formation of colonies in soft agar. OC-NYH cells were treated with chloroquine at 0–290 μM for 20 min followed by addition of camptothecin at 0.3 μM (·····) or dimethyl sulfoxide as control (————). Cells were plated after 24 hr incubation.

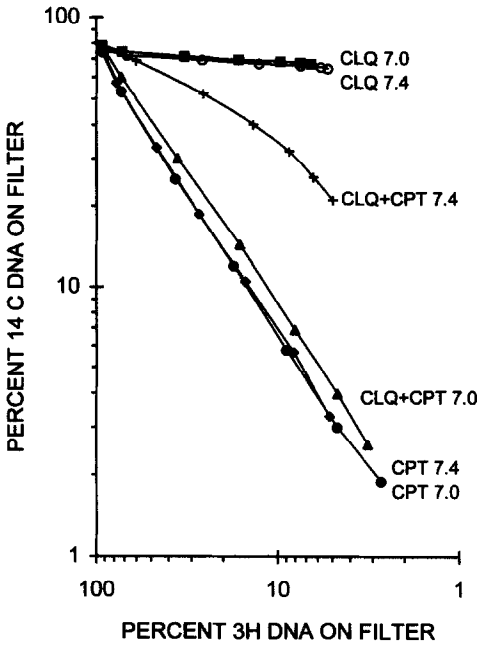

FIG. 6. pH-dependent protection of camptothecin-induced DNA single-strand breaks by chloroquine. DNA single-strand breaks were measured by alkaline elution. [^{14}C]-thymidine labelled OC-NYH cells were incubated at pH 7.0 and 7.4. Cells were treated with (pH 7.4: open circle and cross; pH 7.0, square and triangle) or without 2 mM chloroquine (CLQ) (pH 7.4: diamond; pH 7.0 filled circle) for 10 min followed by an additional 50 min incubation with (cross, triangle, diamond, filled circle) or without camptothecin (CPT) (open circle, square) at 0.5 μM at 37 °C. Internal standard L1210 cells were labelled with [^{3}H]-thymidine. Percent of [^{14}C] and [^{3}H] labelled DNA remaining on filters are plotted logarithmically on the y- and x-axis, respectively. Numbers depicted at each curve indicate extracellular pH value.

induced topoI trapping at pH 6.6 as shown in Fig. 7, lane 6, in contrast to the full protection seen at pH 7.4, lane 3. Additionally, we measured the ability of CLQ to antagonise CPT-mediated cytotoxicity at varying pH (Fig. 8). As prolonged CLQ incubation is quite cytotoxic (see Fig. 5), we used high dose (3.8 mM) CLQ for only 60 min, followed by 24 hr CPT incubation. At pH 7.2, a large fraction of cells treated with both CLQ and CPT survived, corresponding to a protection of more than 2 logs or 7 to 8 cell doublings as compared to treatment with CPT alone. When the pH was lowered to 6.2, CLQ protection was almost abrogated due to the trapping of the weak base CLQ in the acidic extracellular fluid [10]. In contrast, accumulation of [^{3}H]-CPT was not reduced when cells were co-incubated with CLQ at varying pH as low as 6.2. In fact, accumulation of CPT was increased by 20% when treated with CLQ as compared to controls (Fig. 9).

DISCUSSION

In recent years, considerable interest has been shown in topoI targeting agents, as preclinical studies have indicated a lack of cross-resistance to established drugs, e.g. etoposide. Furthermore, response rates in early clinical trials in patients with refractory disease have been promising [4]. Consequently, increasing knowledge on the functions of the enzyme has emerged and several inhibitors of topoI have been identified. It is known that some intercalating agents interfere with the catalytic process of topoI in subcellular systems [5]. In this study, we show that the antimalarial agent CLQ is a catalytic inhibitor of eukaryote topoI, causing inhibition of CPT-mediated cleavable complex formation at a relatively high concentration of CLQ. Moreover, these findings are in accordance with a previous study showing that CLQ inhibits topoI derived from *trypanosoma cruzi* [20]. Further, we demonstrate that inhibition by CLQ of the effects of CPT translates to whole cells. Thus, CLQ inhibits CPT-mediated trapping and single-strand breaks as well as CPT-mediated cytotoxicity. The catalytic cycle of topoI has been divided into 4 different steps. The non-covalent binding of enzyme to the DNA substrate, covalent binding of enzyme and cleavage of single-strand DNA, swiveling or strand passage, and finally rejoining of the DNA strand break [21]. CLQ presumably inhibits the catalytic cycle by intercalating into DNA whereby the enzyme is denied access to its DNA substrate. This notion is strongly corroborated by our finding that CPT-mediated trapping of topoI is antagonised by CLQ in a band depletion assay of whole cells. Likewise, in a subcellular system, it has been observed that the catalytic cycle of topoI is blocked at the step of binding between enzyme and DNA by the minor groove binder distamycin A

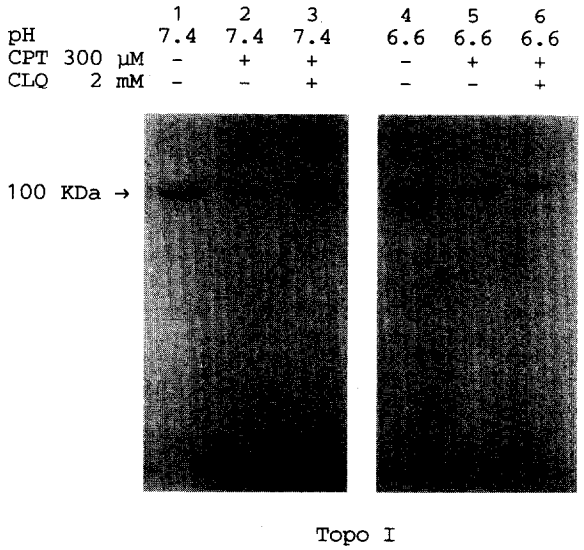

FIG. 7. pH-dependent prevention of camptothecin-mediated trapping of topoisomerase I to DNA by chloroquine in whole cells. TopoI trapping was assessed by a band depletion assay. At pH 7.4 (lanes 1–3) and 6.6 (lanes 4–6), OC-NYH cells were incubated at 37 °C with (lanes 3 and 6) or without (lanes 1, 2, 4, and 5) chloroquine (CLQ) at 2 mM for 15 min followed by an additional 30 min of camptothecin (CPT) incubation at 300 μM (lanes 2, 3, 5 and 6). Control cells were treated with dimethyl sulfoxide only (lanes 1 and 4). 350 mM NaCl nuclear extracts were prepared and topoI was detected by Western blotting. Arrow indicates the position of the 100 kDa molecular weight marker.

[22]. Accordingly, distamycin A and other minor groove binders such as Hoechst 33258 and DAPI (4′,6-diamidino-2-phenylindole) inhibit the formation of CPT-induced DNA lesions [6]. Furthermore, we recently demonstrated that the intercalating anticancer drug aclarubicin antagonises CPT-induced cytotoxicity, damage in genomic DNA and cleavage of liniarized DNA [7].

Our finding that CLQ interferes with the catalytic process of topoI might lead to more selective CPT-based therapy by means of pharmacological regulation. Ideally, such regulation should target cytotoxicity towards tumor cells, leaving normal cells unharmed. Such strategies are currently employed in the clinic using an antagonist to rescue non-malignant tissue, e.g. folinic acid rescue of methotrexate toxicity. Currently, high-dose chemotherapy with bone marrow transplantation is widely used in an effort to circumvent clinical drug resistance, a substantial clinical problem in a variety of malignant diseases. Resistance towards CPT analogs will obviously represent a clinical problem in the future. Experimental resistance is predominantly associated with a reduction in topoI level [23–25], or mutated forms of the enzyme [26–28] leading to the formation of fewer cleavable complexes. In this paper, we present an alternative model for protecting normal tissue against the cytotoxicity of CPT, one exploiting the fact that solid tumors tend to develop an acidic extracellular environment. The principle of the model is to combine a neutral anticancer agent which accumulates in cells in a

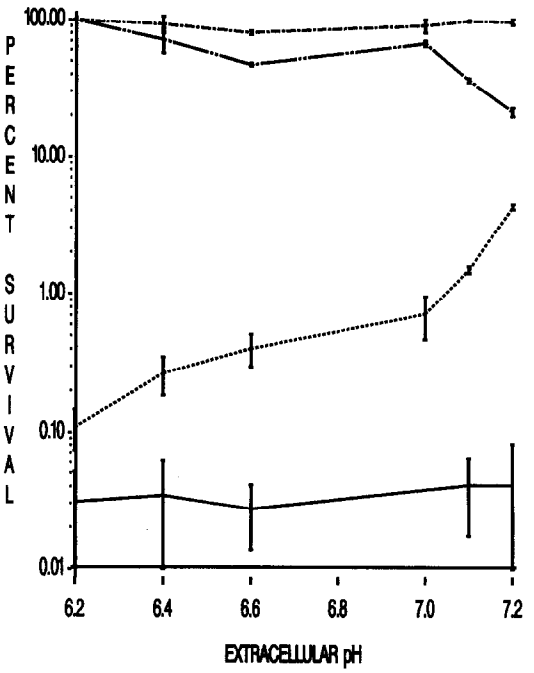

FIG. 8. Antagonism of camptothecin-induced cytotoxicity by chloroquine in a pH-dependent manner. Cytotoxicity was assessed by the formation of colonies in soft agar. OC-NYH cells were treated with chloroquine at 3.8 mM for 60 min at varying pH. Medium was removed and cells were incubated with medium at pH 7.4 supplemented with 0.1 μM camptothecin for 24 hr. Cells treated with dimethyl sulfoxide alone (– · – · – · – · –); cells treated with chloroquine only (· · —— · ·); cells pre-treated with chloroquine followed by camptothecin treatment (· · · · · · · · ·); cells treated with camptothecin only (————).

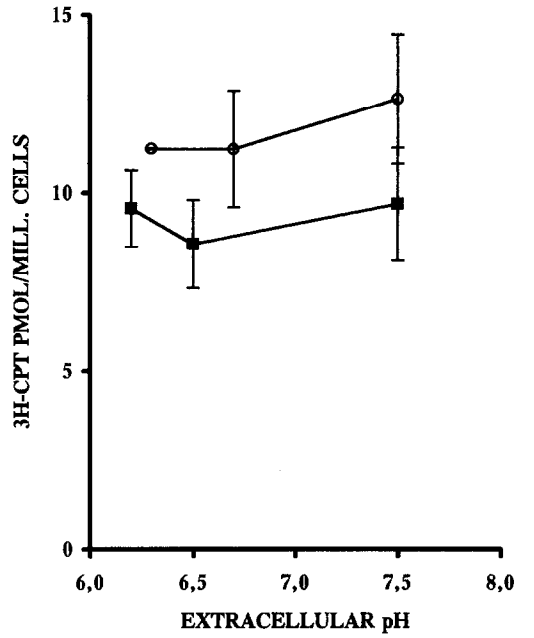

FIG. 9. Accumulation of [^{3}H]-camptothecin with or without co-incubation with chloroquine at varying pH. OC-NYH cells were incubated for 60 min with 5 μM [^{3}H]-camptothecin (CPT) at varying pH in PBS with 5% FCS. Chloroquine (CLQ) at 2 mM was added before CPT. Intracellular concentration of CPT is indicated as picomol per 10^6 cells. Cells treated with [^{3}H]-CPT only (square); cells pre-treated with CLQ (open circle). Error bars indicate two standard deviations.

pH-independent manner with a weak base antagonist which is trapped outside cells at acidic pH [10]. This simple model would have the advantage of protecting all normal tissue at pH 7.4 as well as being relatively inexpensive. Here, we clearly demonstrate that cytotoxicity, DNA strand breaks, topoI nuclear trapping and drug accumulation [10] are strongly correlated to extracellular pH when cells are treated with both CLQ and CPT. This correlation becomes apparent at different drug concentrations and pH values depending on the assay used; e.g., pH 7.0 affords no protection against strand breaks (Fig. 6) whereas the same pH value enables protection of one log in the clonogenic assay (Fig. 8). However, such a discrepancy is not surprising when taking into account that CPT-induced DNA breaks appear within minutes as opposed to the 24 hr incubation time needed for CPT-induced cell kill. Thus, the presented data support the feasibility of the model using CLQ as a pH-dependent topoI catalytic inhibitor in an effort to protect against the cytotoxicity of CPT on cells at pH 7.4 whereas the full effect of CPT is maintained in cells in acidic environments. However, these results are not readily translated into the clinical situation, as the concentrations employed exceed clinical achievable plasma levels following both prophylactic and curative treatment of malaria [29]. The data presented offer CLQ as a lead compound in modulating CPT cytotoxicity, and we are currently testing existing as well as *de novo* synthesised analogs in an effort to identify less toxic compounds.

In conclusion, we demonstrate that the widely used antimalarial agent CLQ is an inhibitor of the topoI catalytic cycle. CLQ antagonises the effect of CPT in cells at neutral extracellular pH, whereas the full effect of CPT is maintained in cells in acidic environments. The presented model offers a strategy for differentially protecting normal tissue at physiological pH against CPT-induced cytotoxicity without reducing the cytotoxic effect of CPT in tumor cells at acidic pH. This principle could serve as a powerful modulator in the future design of cancer regimens.

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