Cellular Pharmacokinetics and Cytotoxicity of Camptothecin and Topotecan at Normal and Acidic pH

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ABSTRACT

pH-mediated conversions in the structure of the topoisomerase (topo) I inhibitors camptothecin (CPT) and its analogues have strong implications for the pharmacokinetics and pharmacodynamics of these novel anticancer agents. Because the cell-penetrating and biologically active lactone isomers predominate at acidic conditions, we have tested if low pH potentiates the cytotoxic and antitumor effects of CPT and its water-soluble derivative topotecan (TPT). In L1210 leukemia cells, rapid initial uptake of radiolabeled CPT and TPT was followed by a gradual release from cells at physiological pH 7.4, whereas high drug levels were maintained in cells at pH 6.2. Steady-state uptake levels of CPT increased proportionally, up to 5-fold, with decreasing pH of the incubating medium (from 7.4 to 6.0). With TPT, a maximum 3-fold increase was observed at pH 6.8 to 6.4. By contrast, the cellular pharmacokinetics of the topoisomerase II inhibitor etoposide (ETP) were independent of the ambient pH. The large increases in intracellular CPT and TPT levels caused only moderate potentiation of cytotoxicity in short-term incubations. Conditions of very low pH ≤5.2 even antagonized the cytotoxicity of the topo I and topo II inhibitors, due to inhibition of DNA synthesis by intracellular acidification. However, in clinically relevant schedules of prolonged exposures at low drug concentration, low pH potentiated the cytotoxicity of CPT and TPT by 2-3-fold. To investigate the effect of local pH in vivo, the basal intracellular pH of 6.8 of RIF-1 tumors was selectively lowered by i.p. injection of the host animals with the mitochondrial inhibitor meta-iodobenzylguanidine (32 mg/kg) and glucose (1.5 g/kg). In accordance with the pH optimum for TPT uptake at pH 6.8 to 6.4, tumor acidification had no effect on the antitumor effect of this analogue. By contrast, the intervention significantly potentiated the response of tumors to CPT. The results indicate that local pH is an important determinant of the cellular pharmacokinetics and the antitumor activity of CPT and analogues.

INTRODUCTION

The cytotoxicity of several conventional and experimental anticancer drugs, notably bioreductive and alkylating agents, is often potentiated by acidic pH (1–3). Tumor-selective acidification has been achieved in animal and human tumors by various interventions. These include the stimulation of the intrinsically higher glycolytic flux of tumors by glucose infusion alone (4) or with coadministration of the mitochondrial inhibitor MIBG5 (5) and the administration of vasoactive drugs like hydralazine (6). The strategic concept of tumor-selective acidification, combined with pH-sensitive (pro)drugs or with drugs that interfere with intracellular pH control (7), is under investigation by several groups (8–10).

Among the pH-sensitive candidate drugs, the topoisomerase I-targeting CPT and its water-soluble derivative TPT hold special promise. Under acidic conditions, the parent drugs exist predominately in the ring-closed lactone form (11, 12), which is the most active configuration for inhibition of topoisomerase I enzyme activity (13). At neutral or alkaline pH, both drugs are converted by hydroxyl-ion-catalyzed hydrolysis into a ring-open carboxylate form (11). Carboxylate CPT and TPT penetrate cells poorly (14), and up to 98% of carboxylate CPT binds to serum proteins at physiological pH (15). These physico-chemical properties predict that CPT and TPT should be much more cytotoxic at conditions of low pH because higher intracellular concentrations of the drugs in the pharmacologically active form may be achieved. The profound urological toxicity of CPT has been, therefore, attributed to increased local bioavailability and maintenance of the biologically active lactone form in an acidic environment (16). Increased cytotoxicity of CPT and TPT at low pH in vitro has been reported from a single study, but this was not correlated with altered drug uptake (17). To our knowledge, there are no reports on the effect of local pH on the cellular pharmacokinetics of topoisomerase I inhibitors nor on potentiation of their antitumor effects after selective lowering of the interstitial pH of tumors in vivo.

We have, therefore, studied the effect of the incubating pH on the kinetics of uptake and retention of radiolabeled CPT and TPT and correlated the alterations in drug accumulation with the cytotoxic potential in clonogenic assays. These studies were performed in mouse L1210 leukemia cells in which the interrelationship between extra- and intracellular pH and the spectrum of sensitivity to various pH-dependent drugs have been amply studied (3). To exclude effects of low pH on drug uptake and activity, unrelated to the specific physico-chemical properties of CPTs, control studies were performed with the topoisomerase II inhibitor ETP. This drug resembles topoisomerase I inhibitors in the ability to form cleavable complexes in DNA and in the requirement for active DNA replication for cytotoxicity (18), but its lactone ring is very slowly hydrolyzed compared with that of topoisomerase I inhibitors. In addition, we tested in pilot experiments whether selective acidification of murine RIF-1 tumors, by treatment of the host animals with MIBG and glucose (5), can improve the antitumor effect of the CPTs. The results reveal that the ambient pH strongly and specifically affects uptake and retention of topoisomerase I inhibitors and that an acidic environment promotes their cytotoxic and antitumor activities.

MATERIALS AND METHODS

Cells and Tissue Culture Methods. L1210 mouse leukemia cells were grown in RPMI 1640, supplemented with 10% FCS, 60 μM β-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml), in a 5% CO2 atmosphere at 37°C. Incubations at different pH were performed in closed culture vials in bicarbonate-free medium, adjusted to the desired pH by addition of 0.5 M lactic acid. Clonogenic survival was assayed by seeding cells in appropriate dilutions in complete RPMI 1640, supplemented with 0.6% carboxymethylcellulose and 20% conditioned medium (3). Macroscopic colonies were counted after 10 days. Absolute plating efficiencies were between 70 and 90% and not affected by short-time incubations in acidified medium. Prolonged incubations at pH 6.2 for 16 h reduced the plating efficiency by maximally 30%. DNA synthesis was monitored by the incorporation of [3H]thymidine into acid-precipitable cell material.

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4 The abbreviations used are: MIBG, meta-iodobenzylguanidine; CPT, camptothecin; TPT, topotecan; ETP, etoposide.

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Drugs and Radiochemicals. TPT was kindly provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA), and [14C]TPT was made available by SmithKline Beecham (The Fryte, Welwyn, United Kingdom). CPT and ETP were purchased from Sigma Chemical Co. (St. Louis, MO). [1H]CPT and [1H]ETP were from Moravek Biochemicals (Brea, CA), and [14C]- and [1H]polyethylene glycol 4000 from Amersham Corp. (Buckinghamshire, England). Stock solutions of CPTs in saline were adjusted to pH 8.0 (carboxylate form) or pH 4.0 (lactone form). MIBG was synthesized as described previously (5) and dissolved in saline.

Drug Uptake Assay. The uptake of [1H]CPT, [14C]TPT, and [1H]ETP was determined without intercurrent washing of cells, as described previously (19). Briefly, cells were suspended in a medium of 37°C (5 × 10^6/ml) containing 14C- or H-labeled drug in tracer amounts (0.5 μCi/ml), supplemented with unlabeled drugs to the desired final molar concentration as indicated. In addition, non cell-penetrating, counterlabeled [1H]- or [14C]polyethylene glycol 4000 was added in equal radioactive concentrations as markers of the extracellular volume. Triplicate samples of 10^5 cells were collected at regular intervals by rapid centrifugation in an Eppendorf centrifuge and dissolved in 0.1 M NaOH. The 3H and 14C radioactivity of drugs and markers in the cell pellets and of 100-μl aliquots of the incubating medium was differentially determined by liquid scintillation counting. Cell-associated drug was calculated in individual samples by subtracting the amount of extracellular drug (estimated from the radioactivity of the extracellular marker) from the total amount of drug in the cell pellet. The median cell volume (12 fl/cell) was calculated from three orthogonal diameters determined with calipers, and body weight was recorded daily. The relative tumor size was calculated in a percentage of that on day 0 (start of treatment; 100%). Tumor responses were pretreatment tumor volume and tested for statistical significance using Student's t test. Toxicity was monitored as severe illness or intercurrent death and by daily measurement of body weight of individual animals.

RESULTS

Pharmacokinetics of CPT and TPT at Different pH. The effect of the incubating pH on the cellular pharmacokinetics of radio labeled CPT and TPT was investigated. Preliminary studies revealed rapid and complete removal of CPT and of 60% of TPT from the cells during three consecutive washing steps with PBS, necessary to remove extracellular radioactivity. Therefore, a rapid assay with nonpenetrating markers of the extracellular volume was used instead of usual washings as described in the previous paragraphs. Fig. 1 shows the effect of the incubating pH on the intracellular drug levels of CPT (Fig. 1A) and TPT (Fig. 1B), both administered in the lactone form. Unlabeled drug was added to the radiolabeled compounds to a final cytotoxic dose of 1 μg/ml. The initial uptake of CPT was rapid and slightly stimulated by low pH, and the peak intracellular CPT concentrations exceeded the extracellular concentration of 2.9 μM by about 3-fold. Subsequently, CPT was rapidly lost from the cells at physiological pH but at a much slower rate at pH 6.2. This characteristic pattern of rapid initial uptake and subsequent pH-dependent release was also observed with TPT (Fig. 1B). However, the initial uptake of TPT at low pH was slightly delayed compared with that at pH 7.4, and peak intracellular TPT concentrations exceeded the extracellular drug level of 2.4 μM only by 1.3-fold. The results in Fig. 1 were similar to those of experiments using [3H]CPT and [14C]TPT in much lower radioactive tracer doses (data not shown), and the uptake characteristics are thus independent of drug concentration.

To confirm the predominant role of the incubating pH in the dynamic changes in drug levels, L1210 cells were first loaded with radiolabeled drug for 60 min at normal or acidic pH. The pH of the incubating media was subsequently converted from 7.4 to 6.2 by the addition of calibrated volumes of 0.5 M lactic acid or vice versa with 0.2 M NaOH. A decrease in the extracellular pH stimulated (re)uptake of CPT, whereas a shift from low to neutral pH accelerated its loss from the cells (Fig. 2A). With TPT (Fig. 2B), the effects of pH reversal were similar but with complex and much delayed kinetics of re-uptake or release compared to CPT. By contrast, intracellular levels of ETP rapidly equilibrated with the extracellular concentration, and neither the rate of uptake nor the steady-state levels were affected by the incubating pH (Fig. 3).

Next, cells were incubated for 60 min with radiolabeled drugs in media of different pH. This incubation time was chosen to estimate the steady-state drug levels in subsequent survival assays. With CPT (Fig. 4), drug levels increased progressively with decreasing pH. At the lowest pH tested, intracellular levels exceeded by about 5-fold those at physiological pH and were about 3-fold above the extracellular concentration. TPT accumulation was also stimulated by low pH but reached a maximum in the pH range 6.8 to 6.4. As expected from the results in Fig. 3, the incubating pH had no effect on the levels of ETP.

pH and Cytotoxicity. To investigate the effects of pH-mediated variations in drug uptake and retention on cytotoxicity, dose-survival...
pH 6.2, as is shown for CPT in Fig. 5. Decreased cytotoxicity at pH 6.2 was also observed with the topoisomerase II inhibitor ETP (data not shown). Topoisomerase I and topoisomerase II inhibitors both require active DNA synthesis for their cytotoxic activity (18, 20). We, therefore, tested whether very low pH antagonized the cytotoxicity by inhibition of the rate of DNA synthesis. Previous studies have indicated that incubation of L1210 in a medium of pH 6.2 is accompanied by a rapid lowering of the intracellular pH to about 6.7 (3), which is critical for most metabolic processes (7). Fig. 6 shows that the incorporation of [3H]thymidine dropped markedly in cells incubated for 4 h at pH ≤ 6.2, i.e., the condition that affords protection against topoisomerase I and II inhibitors. During prolonged, 16-h incubations, [3H]thymidine incorporation was more proportionally inhibited with decreasing pH. In the latter experiments, inhibition of DNA synthesis was also evident from reduced cell division and a decrease in the fraction of S-phase cells, recorded by DNA flow cytometry (data not shown). The abrupt inhibition in short-time incubations suggest that a critical intracellular acidification to pH 6.7 by an extracellular pH of 6.2 strongly affects the rate of ongoing DNA synthesis. The additional inhibition of [3H]thymidine incorporation at higher pH values during prolonged exposures probably reflects the reduced progression to S-phase. During short-time incubations at pH values that did not effect the rate of DNA synthesis (pH 7.4 to 6.4), only a marginal increase in the cytotoxicity of CPT and TPT, but not of ETP, was observed (data not shown). Moreover, potentiation of CPT cytotoxicity was only observed in low drug doses (<0.5 μg/ml) and absent at higher dose (>1.0 μg/ml). This finding is consistent with the saturating shape of the survival curve (Fig. 5), implying that stimulated drug uptake will not result in a further increase in the level of cell kill. However, prolonged exposure at low dose (0.05 μg/ml), reflecting the clinically applied schedules of continuous infusion or frequent administration, revealed that CPT and TPT were both progressively more cytotoxic with decreasing pH (Fig. 7). The dose-reduction factors, estimated from the log-linear part of survival curves after 16 h exposure at physiological pH, indicated 2—3-fold potentiation of drug action at pH 6.2. This level of potentiation was less than the 4-fold increase predicted from the stimulated uptake (Fig. 4), probably because of simultaneous inhibition of cell cycle progression.
By contrast, the two isomers of CPT were each more effective on tumors of MIBG/glucose-treated animals (Fig. 8, C and D). Pretreatment caused an increase in tumor growth delay from 1.4 ± 0.2 days to 2.1 ± 0.2 days (P = 0.05) with carboxylate CPT and from 2.6 ± 0.3 to 4.5 ± 0.4 days (P = 0.009) with the parent drug. None of these schedules caused acute toxicity to the hosts, i.e., intercurrent death or weight loss exceeding 10% of total body weight.

DISCUSSION

CPT analogues are a promising class of anticancer drugs, targeted against topoisomerase I enzymes (16, 23). Whereas the parent compound CPT has been abandoned from clinical studies because of unpredictable toxicity, several derivatives are currently under clinical investigation. These include the water soluble, semisynthetic compound TNP. The level of topoisomerase I enzymes (24), the fraction of S-phase cells (18, 25), and the capacity of P-glycoprotein- or multidrug resistance-mediated drug export (26, 27) have all been identified as important factors in the sensitivity to CPTs. Relatively little is known, however, about the effect of pH-mediated structural changes on the cytotoxicity and antitumor capacity of these drugs.

Theoretically, there are several arguments for an important role of intra- and extracellular H⁺-ion activity in the cytotoxicity of CPTs. In vitro DNA relaxation studies have indicated that the ring-closed lactone, which predominates at increased H⁺-ion concentrations, is biologically most active (13). Administration of the hydroxy acid sodium salt of CPT, however, still causes toxic and antitumor effects in animal tumors (17, 28), and clinical studies have indicated that the pharmacodynamics of TNP are more consistent with the plasma AUC of total drug than with that of the lactone form alone (29). These observations have been interpreted as proton-catalyzed conversion of carboxylate drug into the lactone form, close to or inside the target cells. In addition, the ring-closed configurations of CPT and TNP are 4 h more lipophilic and electroneutral in case of CPT, promoting their uptake in an acidic environment (14). Finally, hydroxylate CPT but not TNP binds to serum proteins (15, 30), and CPT is, therefore, less efficient in vivo than TNP because of reduced bioavailability.

Using a rapid and versatile assay, the cellular pharmacokinetics of
The reason for these differences is poorly understood, especially for the higher UP levels observed immediately after acidification compared with those after 60 min loading at physiological pH. A role of transient cellular injury by the addition of lactic acid and sodium hydrochloride cannot be excluded, however. Moreover, intracellular CPT levels at steady state increased proportional with decreasing pH but reached a maximum in case of TN' at pH 6.8 to 6.4 (Fig. 4). The pH optimum in TN' uptake can be plausibly explained by an adverse effect of very low pH, causing increased protonation of the dimethyl amino groups of TPT, which lowers its lipid partitioning capacity (14), as has been reported for some other drugs as well (3, 31).

Compared with the large, 3–5-fold stimulation of drug uptake, the cytotoxicity of TPT and CPT was only moderately potentiated during short-term incubation at decreased pH, most probably because of the saturating characteristics of the dose-survival curve and of rapid release of drug after plating of cells. At pH 6.2 or lower, there was even antagonism of drug action, not only of TPT and CPT but also of ETP, as reported previously for this drug (31). These findings contrast with the strong potentiation of several other, mostly alkylating, drugs in L1210 cells by low pH (3). However, the decreased cytotoxicity of the topoisomerase I and II inhibitors at pH 6.2 was coincident with a strong decline in the rate of DNA synthesis, and active DNA synthesis is essential for the development of lethal double strand breaks after stabilization of cleavable complexes by topoisomerase inhibitors (18, 20). By contrast, a marked, 2–3-fold potentiation by increased acidity was observed during prolonged incubation at low drug concentration (Fig. 7), i.e., the in vitro correlate of continuous infusion or frequent administration of CPTs in the clinic (29). In these experiments, the level of potentiation is the balance of promoted drug uptake and reduced sensitivity to the drugs by simultaneous inhibition of DNA synthesis. Obviously, the net outcome of low pH-stimulated cytotoxicity of CPTs is complex and dependent on specific conditions of extra- and intracellular pH, the particular analogue, and the treatment schedule.

Although admittedly preliminary, the results of the animal experiments were remarkably consistent with those of in vitro drug uptake studies. TPT was not potentiated by the MIBG/glucose intervention (Fig. 8B). This is in agreement with the observed optimum for TPT uptake in vitro at pH 6.8, i.e., the basal pH of RIF-1 tumors. However, tumors with a neutral interstitial pH may be well be potentiated to TPT by acidification. MIBG/glucose treatment did potentiate the antitumor activity of the two isomers of CN' (Fig. 8, C and D). These findings are consonant with stimulated initial uptake and superior retention of the parent drug at pH 6.2 (Figs. 1A and 4) and with rapid stimulation of the (re)uptake of carboxylate CPT in an acidic environment (Fig. 2A). Although a low interstitial tumor pH could also locally convert carboxylate TPT into the active and cell-penetrating...
lactone form, the delayed kinetics of TPT re-uptake of about 30 min (Fig. 2B) are probably not matching with the rate of tumor perfusion to achieve detectable potentiating of this drug. It could be argued that the pharmacokinetics studied in leukemic cells in vitro inadequately represent that of RIF-1 fibrosarcoma cells, used in the animal studies. Although basically true, it is of note that the effects of ambient pH on drug uptake could all be attributed to the specific physico-chemical properties of the drugs and not to cellular factors.

The present animal experiments probably failed to reveal the full potency of tumor acidification on the antitumor activity of CPT. Because of rapid inactivation of circulating drug and the S-phase dependency of their cytotoxic action, continuous infusion or frequent administration of CPTs is much more effective than the single bolus injection that was applied in the present studies (20, 29). The in vitro studies (Fig. 7) indicated that the effect of low pH was indeed most marked in a clinically relevant schedule. In addition, the estimated 2–3-fold potentiation at pH 6.4 in vitro significantly underestimates the effect of low pH in tumors cells which, unlike tissue culture cells, resist inhibition of DNA synthesis by intracellular acidification. Finally, the arbitrary schedule of MIBG/glucose treatment followed by drug administration 3 h later may be suboptimal. Because an acidic environment promoted both initial uptake and retention (Fig. 1), other schedules or even a reversed sequence could be more effective. It is clear that more studies are needed to fully appreciate the clinical value of basal tumor pH and its manipulation in the therapeutic efficacy of various CPT analogues.

In summary, the present studies are the first to account for the complex cellular pharmacokinetics of topoisomerase I inhibitors and the profound effect of local pH on drug uptake and retention. Although dominated by pH-dependent interconversions between lactone and carboxylate isomers, the uptake of CPT and TPT was also significantly modulated by structural differences between both inhibitors. Low intratumoral pH, either basal or induced by appropriate pharmacological interventions, would seem an important determinant of the antitumor activity of CPTs.

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