Cancer Chemother Pharmacol (2000) 46: 263–271

ORIGINAL ARTICLE

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Camptothecin analogues with enhanced antitumor activity at acidic pH

Received: 12 August 1999 / Accepted: 14 April 2000

Abstract *Background*: Camptothecin (CPT) is a specific inhibitor of the nuclear enzyme topoisomerase I, which is involved in cellular DNA replication and transcription. Topoisomerase I is therefore an attractive target for anticancer drug development, and two analogues of CPT, topotecan (TPT) and irinotecan (CPT-11), have demonstrated significant antitumor activity in the clinic. This activity is limited, however, by lability of the CPT E ring lactone, which forms the inactive hydroxy acid at physiological pH. The reaction is reversible at acidic pH, which provides a rationale for selectivity, because many solid tumors create an acidic extracellular environment while maintaining a normal intracellular pH. *Purpose*: To exploit the tumor-selective pH gradient to improve the efficacy of CPT-based chemotherapy. *Methods*: CPT analogues were evaluated by growth inhibition assay in three human breast cancer cell lines that had been adapted to in vitro culture at acidic pH versus the respective cells cultured at physiological pH. The MCF-7, MDA-MB-231, and MCF-7/hc cell lines represent the hormone-dependent and hormone-independent stages of the disease, and a MCF-7 variant that is resistant to the alkylating agent 4-hydroperoxycyclophosphamide (4-HC), respectively. Antiproliferative activity of SN-38 (the active metabolite of CPT-11), and TPT was compared to that of CPT and two CPT analogues, 10,11-methylenedioxy-CPT (MDC), and the alkylating derivative, 7-chloromethyl-10,11-MDC (CMMDC). *Results*: In general, MDC was the most potent and TPT or CPT the least potent analogue, regardless of pH. However, if the comparison was based on magnitude of potentiation by pH, a different rank order emerged. CPT was modulated 4-fold; MDC, SN-38, and TPT were each modulated 5- to 6-fold, while the activity of CMMDC was increased 10- to 11-fold by acidic pH in MCF-7 lines, and 65-fold in MDA-MB-231 cells. Thus MDC was the superior CPT analogue based on potency, but CMMDC was the best candidate for pH modulation. Drug specificity was also observed. While the alkylating agent, 4-HC, was 2- to 3-fold more active at acidic pH, modulation was not observed for 5-fluorouracil, doxorubicin, or paclitaxel. Preliminary mechanism studies indicated that pH modulation of CPT analogues was directly correlated to intracellular levels of glutathione. In addition, protein-associated DNA strand breaks were more rapidly induced at acidic pH. *Conclusion*: These results suggest that CPT-based drug development and resulting chemotherapy could benefit from evaluation of differential activity at acidic versus physiological pH. Analogues have been identified that could have improved therapeutic indices based on the pH gradient that selectively exists in human tumors.

Key words Camptothecin · pH · Glutathione · In vitro screens · Drug development

Abbreviations SN-38 (7-Ethyl-10-hydroxy camptothecin) · *CMMDC* 7-Chloromethyl-10,11-methylenedioxy camptothecin · *CPT* Camptothecin · *CPT*-11 Irinotecan · *TPT* Topotecan · *GSH* Glutathione · *4-HC* 4-Hydroperoxycyclophosphamide · *MDC* 10,11-Methylenedioxy camptothecin · pH_i Intracellular pH · pH_e Extracellular pH

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Introduction

Recently there has been renewed interest in topoisomerase I as an antitumor target, due in large part to the

success of topotecan (TPT) and irinotecan (CPT-11) in the clinic [11, 19]. Accordingly, many second-generation CPT analogues are now in clinical development, including karenitecin, 9-nitro-CPT, and 9-amino-CPT. Most new CPT analogues have been developed to optimize drug interaction with DNA, topoisomerase I, or both, to achieve more stable, potent compounds that produce persistent DNA damage. In general, CPT analogue development has not focused on ways to improve selectivity for human tumor cells, despite advances in our understanding of tumor biology. For example, solid tumors are known to acidify their extracellular environment by releasing lactic acid, a by-product of anaerobic glycolysis. This effect leads to a pH gradient that exists selectively in tumor cells and can have a dramatic effect on drug uptake and activity. However, virtually all in vitro screens for antiproliferative activity, including that utilized by the National Cancer Institute [15], evaluate tumor cells cultured at physiological pH. Extracellular pH could have a significant impact on screening results, particularly for analogues of CPT that retain the pH labile E ring lactone. Indeed, previous work by Teicher and coworkers [25] in EMT-6 murine mammary carcinoma cells demonstrated that brief (1 h) exposures to high concentrations of both CPT and TPT in vitro were over 30-fold more cytotoxic at pH 6.45 than at normal pH. Similarly, Gabr et al. [4] found a 2- to 3-fold increase in activity of CPT and TPT in L1210 leukemia cells exposed for 16 h at pH 6.2. This increase in activity corresponded to a comparable increase in intracellular drug concentration. In contrast, Vukovic and Tannock [27] found that antiproliferative activity of TPT was significantly reduced at pH 6.5 versus pH 7.4, when either EMT-6 or human MGH-U1 cells were exposed for 24 h. In addition, agents that cause intracellular acidification also reduced TPT cytotoxicity. The reason for this discrepancy is not clear; however, all three studies were conducted under acute acidification conditions. Tumor cells adapted to growth at acidic pH exhibit an altered pattern of gene expression that can affect drug response. For example, in human melanoma cells adapted to culture at pH 6.7, the heat shock proteins, HSP70 and HSP27 were upregulated. This effect was correlated with cellular resistance to cisplatin and to hyperthermia [28]. In the current work, human breast tumor cells were adapted to growth in vitro at pH 6.8. When CPT analogue antiproliferative activity in these cells was compared to that at normal pH, a new rank order emerged. One analogue in particular (CMMDC) was much more potent at acidic pH. This potency was tightly correlated to depletion of intracellular glutathione (GSH) and to rapid onset of DNA damage. These results suggest that a screening strategy that more closely reflects human tumor biology could generate new candidates for antitumor drug development.

Materials and methods

Reagents and drugs

TPT, SN-38, 10,11-methylenedioxy-CPT (MDC), and 7-chloromethyl-10,11-MDC (CMMDC) were synthesized by published methods [10, 12, 21, 29]. Formulated doxorubicin, 5-fluorouracil, and paclitaxel were obtained from Pharmacia & Upjohn (Kalamazoo, Mich., USA), Pharmacia (Kalamazoo, Mich., USA), and Bristol-Meyers Squibb (Princeton, N.J., USA), respectively. 4-Hydroperoxycyclophosphamide (HC) was a generous gift from Dr. Susan Ludeman (Duke University, Durham, N.C., USA).

Cell culture

The MCF-7 human breast cancer cell line was obtained from Dr. Alex Miron (Duke University, Durham, N.C., USA). MCF-7/hc cells were obtained from Dr. Beverly Teicher (Dana Farber Cancer Institute, Boston, Mass., USA), while MDA-MB-231 cells were purchased from the American Type Culture Collection (Rockville, Md., USA). MCF-7 lines were grown in DMEM basal medium (Sigma Chemical, St. Louis, Mo., USA) containing 2 g/l glucose. MDA-MB-231 cells were cultured in RPMI-1640 medium (GIBCO, Grand Island, N.Y., USA). Both growth media were supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA). The pH of the respective media was adjusted by varying the concentration of bicarbonate buffer and adding sodium chloride to maintain equivalent osmolarity. Cell lines were passaged at least twice in low pH medium before assessment of chemosensitivity.

In vitro cytotoxicity assay

A calcein-AM metabolic assay was used to assess antiproliferative activity. Viable cells contain a cytoplasmic esterase that converts the cell-permeant and nonfluorescent substrate calcein-AM to a highly charged, and thus retained, fluorescent product (calcein). Dead or dying cells fail either to metabolize the substrate or to retain the product. Tumor cells were plated at 5,000–10,000 cells per well in 96-well microtiter plates (Corning Costar, Acton, Mass., USA), and incubated in 5% CO_2, 95% humidified air at 37 °C. After 24 h drugs were added at seven concentration levels to quadruplicate wells in a final volume of 200 µl. To compensate for pH effects on growth kinetics tumor cells were exposed to agents for two cell doublings and then to drug-free medium for one cell doubling. Culture medium was replaced with phenol red-free Hank's balanced salt solution containing 5 µg/ml calcein-AM (Molecular Probes, Eugene, Ore., USA). Cells were incubated for 1 h at 37 °C, and the fluorescence quantitated in a Bio-Tek FL600 fluorescence microplate reader (Bio-Tek, Winooski, Vt., USA). Dose-response curves were constructed using Table-Curve 2D software (SPSS, Chicago, Ill., USA), and used to determine the median effective concentration (IC_{50}), the drug concentration that inhibits tumor growth to 50% that of untreated control cells.

Assay for DNA-protein cross-links

Agent-induced DNA-protein cross-links were measured by a modification of the potassium SDS precipitation assay of Rowe et al. [20]. Briefly, tumor cells ($0.5–1 \times 10^5$/ml per well in a 24-well microplate) were labeled with [^{3}H]thymidine (2 µCi/ml) for 24 h and then cultured in label-free medium for 2–4 h prior to drug exposure. Cells were washed with DPBS, lysed for 1 min at 65 °C on a plate shaker with 0.25 ml lysis buffer (10 mM Tris-OH, pH 8.0, 5 mM NaEDTA, 1.25% sodium dodecyl sulfate, 0.4 mg/ml calf thymus DNA), then transferred to 1.5 ml microcentrifuge tubes, and stored at −80 °C. Lysates were thawed, reheated (65 °C for 10 min), and the DNA sheared by ten passes through a 1-ml

pipet tip. SDS-protein complexes covalently linked to DNA fragments were precipitated by addition of 0.2 volumes of 2.5 M potassium chloride, followed by incubation on ice for 10 min. The pellets were collected by centrifugation in a Sorvall RC-5C centrifuge at 5000 *g* for 15 min at 4 °C. Supernatants were removed and the pellets were resuspended in 0.5 ml wash buffer (10 mM Tris-OH, pH 8.0, 1 mM Na EDTA, 100 mM KCl, 0.1 mg/ml calf thymus DNA), incubated at 65 °C for 10 min, cooled on ice for 10 min, and centrifuged as above. This wash step was repeated two additional times, then the final pellet was dissolved in 0.5 ml water at 65 °C for 10 min, and transferred to a counting vial containing 4 ml Ultima Gold MV scintillation cocktail (Packard Instrument, Meridean, Conn., USA). Radioactivity was quantitated in a Packard Model 1500 liquid scintillation counter. The percentage of DNA-protein cross-links was computed as the ratio of CPM precipitated to the total CPM in the lysate.

Intracellular glutathione assay

The method of Millis et al. [14] was used to quantitate intracellular GSH. Briefly, cells were harvested, counted, treated with 5 mM monobromobimane, and extracted with 0.5 N perchloric acid. The resulting GSH conjugates were resolved by reverse phase high performance liquid chromatography on a Zorbax C18 column (Waters, Milford, Mass., USA), using an aqueous mobile phase of 18% methanol in 0.25% glacial acetic acid, pH 3.9, and a gradient of 0–30% acetonitrile. Bimane-associated fluorescence was detected with excitation and emission filters of 350 and 470 nm, respectively. Results were expressed as total femtomoles of GSH per cell.

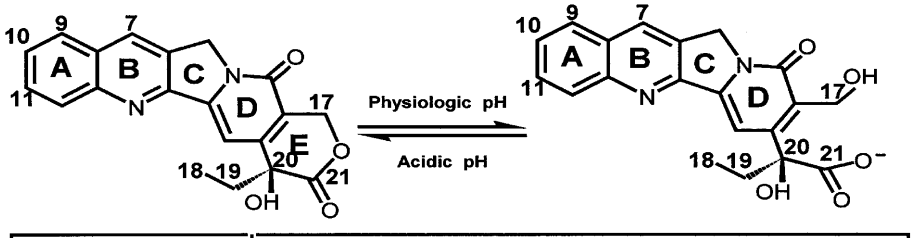
Analysis of CPT analogue lactone versus carboxylate forms

The relative amounts of lactone and carboxylate forms of the CPT analogues formed at pH 6.8 and 7.4 were determined by an HPLC method. The analogues were diluted in DMSO to concentrations between 0.5 and 1 mM before dilution to 1 μM into 0.05 M phosphate buffer at pH 6.8 or 7.4. This reduces aggregation of the analogues in aqueous solution, which slows the ring opening [16]. Aggregation was particularly evident with CMMDC, which must be diluted to at least 0.5 mM in DMSO before further dilution in the buffers. The amount of lactone and carboxylate forms of the CPT analogues was determined by a modification of the HPLC method as described by Warner and Burke [30]. Solvents used were A, acetonitrile, and B, 2% triethylamine adjusted to pH 5.5 with glacial acetic acid. The various analogues were eluted by changing the initial conditions and gradient profile. For MDC and CMMDC the initial conditions were: 25% A, 75% B. This was increased linearly to 40% A, 60% B over 5 min. Over the next minute the gradient was changed to 100% A, where it remained for 4 min before returning to the initial conditions. For SN-38 the initial conditions were 35% A, 65% B, which was unchanged for the first 6 min, the solvent was then changed linearly to 100% A over the next minute. The column was washed with 100% A for 4 min before returning to the initial conditions. For TPT initial conditions were 15% A, 85% B, which increased linearly to 30% A, 70% B, over 3 min. The solvent was then increased to 100% A over the next 2 min, where it remained for a further 2 min before returning to the initial conditions. In each case the carboxylate form eluted within 2–3 min followed by the lactone form in 6–8 min.

The UV spectrum of the column eluents was monitored between 310 and 410 nm using the Waters 996 photodiode array detector. Since the position of the UV absorbance shifts slightly with ring opening, the chromatograms were obtained at the wavelength corresponding to the absorbance maxima (λ_{max}) for each compound. The area beneath the peak at the λ_{max} was used to calculate the relative amount of lactone and carboxylate form. The relative amounts calculated in this way assume that the molar absorptivity coefficient for the lactone and carboxylate forms are similar. This is not an unreasonable assumption [16]. However, since the carboxylate and lactone forms elute from the column and arrive at the detector under different solvent conditions, the molar absorptivity coefficient may change with solvent. To reduce the error in this assumption we compared the ratios of lactone to carboxylate at pH 7.4 and 6.8 rather than the absolute values.

Results

Figure 1 presents the structure for CPT and selected CPT analogues, including the two CPTs in clinical use, TPT and CPT-11, and SN-38, the active metabolite of CPT-11. These analogues were compared to a newer CPT, MDC, which contains a 10,11-methylenedioxy substitution bridging the C10 and C11 positions. The chloromethyl group at C7 in CMMDC adds an alkylating functionality to MDC that produces persistent DNA damage [18, 26]. While these modifications have improved potency and solubility, all the analogues have



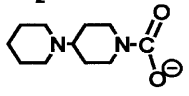
	C7	C9	C10	C11	C20
CPT	H	H	H	H	OH
TPT	H	$(CH_3)_2NHCH_2$	OH	H	OH
CPT-11	C_2H_5	H		H	OH
SN-38	C_2H_5	H	OH	H	OH
CMMDC	CH_2Cl	H	$-O-CH_2-O-$		OH
MDC	H	H	$-O-CH_2-O-$		OH

Fig. 1 Structure of CPT analogues. Chemical structures for the clinical agents TPT and CPT-11 are compared to the parent, CPT, to SN-38, the active metabolite of CPT-11, and to the newer analogues, MDC and CMMDC. The E ring lactone is converted to the inactive hydroxy acid at physiological pH, but can be reactivated under acidic conditions

an unstable lactone on the E ring that forms the inactive hydroxy acid at physiological pH. Since this reaction is reversible, CPTs should be activated in an acidic environment, such as that surrounding solid tumors in vivo. To test this hypothesis we adapted three human breast tumor cell lines to growth at acidic pH. MCF-7 and MDA-MB-231 cells are models for the hormone-dependent and independent forms of the disease, respectively. In addition, we evaluated the effect of pH on chemosensitivity of MCF-7 cells that were selected in vitro for resistance to the alkylating agent 4-HC.

Acute exposure to acidic conditions can cause inhibition of DNA synthesis [4], which in turn could impact sensitivity to agents such as topoisomerase inhibitors that are active in S phase. Therefore, it was important to first determine whether adaptation to acidic pH could affect the growth rate of breast tumor cells in vitro, and thereby account for any potentiation of CPT analogue activity. We found that each of the breast tumor cell lines could be adapted to growth in medium with pH as low as 6.6. A representative example of the growth kinetics in 96-well microplates for the three breast tumor cell lines is shown in Fig. 2. Tumor cells adapted to culture at pH 6.8 grew at equal or slightly slower rates than those at physiological pH. Doubling times were 25 h for MCF-7/wt and 24 h for MCF-7/hc cells at pH 7.4, versus 27 and 29 h for the respective cells grown at pH 6.8. The growth rate of MDA-MB-231 cell lines at the two pH conditions was indistinguishable, with a doubling time of 50 h. While differences in growth rates were minor, subsequent potentiation experiments were adjusted so that drug exposure and recovery periods were determined by cell doubling time rather than time in culture.

Since the E ring lactone of CPTs is more stable at acidic pH, we reasoned that tumor cells adapted to a low pH growth medium would be more sensitive to growth inhibition by these agents. We found the CPT analogues were all more active at acidic pH than at physiological pH (Table 1). Based on the IC_{50} endpoint, MDC was the most potent CPT while TPT was the least. The rank order of activity was similar in the MCF-7 cell lines with MDC > CMMDC = SN-38 = CPT > TPT (Table 1). The order of activity differed in MDA-MB-231 cells. At pH 7.4, the order was MDC >> CMMDC = SN-38 > TPT = CPT, while at pH 6.8 it was MDC = CMMDC > SN-38 > TPT > CPT. However, the ranking was markedly different if the comparison was based on magnitude of potentiation by pH. CPT was modulated 4-fold, MDC, SN-38 and TPT were each modulated 5- to 6-fold, while the activity of CMMDC was increased 10- to 11-fold by acidic pH in MCF-7 lines, and a remarkable 65-fold in MDA-MB-231 cells (Table 1 and Fig. 4). Thus, MDC is the superior analogue based on potency alone, but CMMDC is the best candidate for enhancement based on pH modulation.

To further characterize the effect of pH on CPT activity, we evaluated potentiation as a function of pH. Potentiation was observed at all acidic pH values tested (Fig. 3), with an optimum at pH 6.8. A further decrease in acidity either gave no increase in potentiation or, in the case of CMMDC, caused a decrease in the effect. Cells adapted to culture at pH 6.6 exhibited a retarded growth rate and significantly lower protein content, which could have affected response to CPTs.

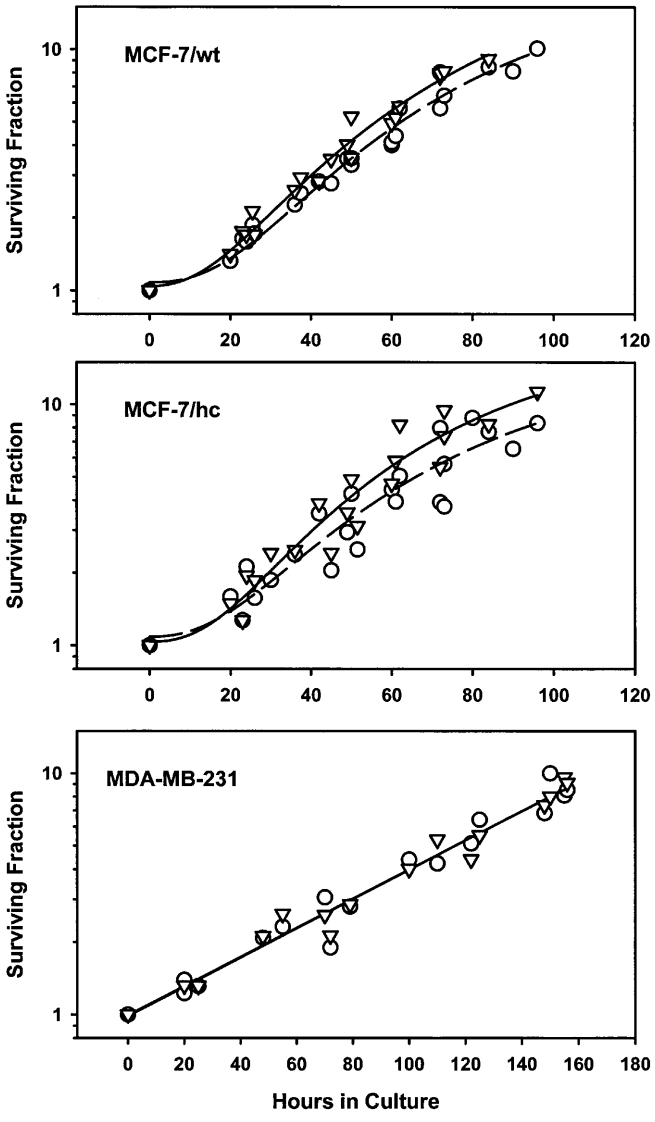


Fig. 2 Growth kinetics of human breast cancer cell lines in microplates at physiological versus acidic pH. Breast cancer cell lines were cultured for at least three passages in vitro prior to assessing growth kinetics as described in the text. *Data points* Average number of nuclei from three replicate microplate wells. The results represent composite data from three to five individual experiments per line for cells cultured at pH 7.4 (*triangles*), or pH 6.8 (*circles*). *Lines* are regression curve fits for the data at pH 7.4 (*solid*) versus pH 6.8 (*dashed*)

We next asked whether the pH effect was selective for topoisomerase I inhibitors. We examined potentiation of the topoisomerase II inhibitor doxorubicin; the DNA antimetabolite 5-fluorouracil, the alkylating agent 4-HC, and the antimitotic agent paclitaxel (Table 2 and Fig. 4). Only 4-HC was more active at acidic pH. The IC_{50} for

Table 1 Potentiation of camptothecin analogues by acidic pH in human breast tumor cell lines. Antiproliferative activity of camptothecin analogues was assessed in breast tumor cell lines representing early stage, late stage, and drug-resistant forms of the disease. Cells were exposed to agents for two cell doublings, and cultured in drug-free medium for an additional doubling prior to assay for surviving, viable cells as described in the text. Data are the mean and standard error from three replicate experiments. Note the difference in rank order when fold potentiation by acidic pH is used as the endpoint

Cell line	Agent	pH 6.8 IC_{50} (nM)	Relative activity[a]	pH 7.4 IC_{50} (nM)	Relative activity[b]	Fold potentiation[c]
MCF-7/wt	MDC	96 ± 16	1.0	517 ± 58	1.0	5.4
	CMMDC	213 ± 41	2.2	2145 ± 134	4.1	0.1
	SN-38	325 ± 64	3.4	2220 ± 186	4.3	6.8
	CPT	416 ± 76	4.3	1498 ± 231	2.9	3.6
	TPT	1038 ± 45	10.8	5705 ± 294	11.0	5.5
MCF-7/hc	MDC	65 ± 10	1.0	329 ± 40	1.0	5.0
	CMMDC	127 ± 4	2.0	1361 ± 49	4.1	10.7
	SN-38	156 ± 31	2.4	1192 ± 165	3.6	7.6
	CPT	303 ± 32	4.7	1289 ± 74	3.9	4.3
	TPT	605 ± 69	9.3	3136 ± 253	9.5	5.2
MDA-MB-231	MDC	21 ± 5	1.0	163 ± 24	1.0	7.6
	CMMDC	23 ± 7	1.1	1526 ± 253	9.3	65.3
	SN-38	144 ± 11	6.7	993 ± 74	6.1	6.9
	TPT	571 ± 92	26.6	2955 ± 118	18.1	5.2
	CPT[d]	826 ± 163	39.3	2895 ± 566	17.8	3.5

[a] Activity normalized to the most potent agent against the respective cell line at pH 6.8
[b] Activity normalized to the most potent agent against the respective cell line at pH 7.4
[c] IC_{50} at pH 7.4 divided by the respective IC_{50} at pH 6.8
[d] Activity evaluated in DMEM containing 10% fetal bovine serum

4-HC dropped 3-fold in MCF-7 and MDA-MB-231 cell lines at pH 6.8. Moreover, there was also a modest increase in activity of 4-HC in 4-HC resistant MCF-7. The MCF-7/hc line was derived in vitro and was reported to be 9-fold resistant to 4-HC [3]. Our results indicate 3-fold resistance to 4-HC with 5-fold cross-resistance to doxorubicin at physiological pH. MCF-7/hc cells cultured at acidic pH are somewhat more resistant to these agents (4- and 6-fold, respectively). There is little to no cross-resistance to paclitaxel or 5-fluorouracil at either pH, while there is slight collateral sensitivity to the CPT analogues.

One possible explanation for the increase in CPT analogue activity at acidic pH is the increased stability of the active E ring lactone form under these conditions. Consequently we investigated E ring opening of the CPT analogues in phosphate buffer at pH 6.8 versus 7.4 after 48 h incubation at 37 °C. The results, summarized in Table 3, indicate that under conditions in which there are no competing equilibria of lactone and carboxylate forms for serum protein binding or lipid partitioning, there could be three to four times as much lactone form at pH 6.8. Moreover, our preliminary survey of pH potentiation of selected CPT analogues in other tumor types has indicated that antiproliferative activity is not affected by acidic pH in cultures of the human prostate tumor cell lines, PC3 and DU145 (D. Adams, unpublished observations). Thus stability of the CPT lactone by acidic pH may account for some but not all of the potentiation observed in tumor growth inhibition assays.

Intracellular GSH is known to be a determinant of sensitivity to alklyating agents and is a general mechanism for drug detoxification [5]. Since the alkylating CPT, CMMDC, and 4-HC were both potentiated by acidic pH, we measured the levels of intracellular GSH in our breast tumor cell lines as a function of extracellular pH. Figure 5A demonstrates that there is a tight correlation between intracellular GSH and sensitivity to CMMDC in MCF-7/wt or MDA-MB-231 cells adapted to growth at pH 6.6 to pH 7.4. In this case an 8-fold increase in sensitivity to CMMDC was associated with a 61% decrease in intracellular GSH. Moreover, MCF-7/hc cells, which express elevated GSH as a mechanism of resistance to alkylating agents, were also sensitized to CMMDC by growth at acidic pH. Similar correlations were observed with MDC, SN-38 and 4-HC (data not shown). Only sensitivity to TPT appeared to be largely independent of intracellular GSH. Increased sensitivity of breast tumor cells to CMMDC was also reflected in the induction of protein-associated DNA strand breaks, a hallmark of DNA damage produced by inhibitors of topoisomerase (Fig. 5B). Thus at 24 h of exposure, 1 μM CMMDC produced four times the DNA damage at pH 6.8 than at physiological pH in MCF-7/hc cells.

Discussion

Tumors utilize both aerobic and anaerobic glycolysis, which ultimately leads to acidification of the local environment. Recent advances in methodology have further enabled discrimination of intracellular tumor pH (pH_i) from that of the extracellular space (pH_e). These studies have established an important rationale for selective attack on tumor cells: the tumor pH gradient [6]. While the average pH_i for normal and tumor tissues is comparable (7.2), pH_e can range from 6.8 to 7.2 in solid

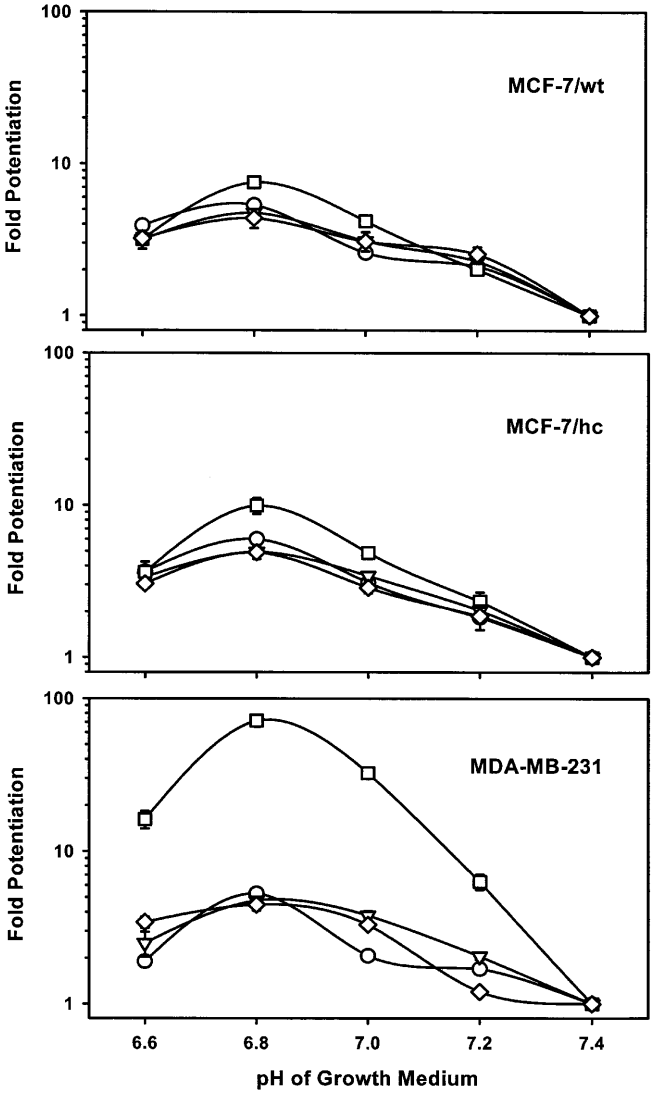


Fig. 3 pH optimum for antiproliferative activity of CPT analogues at acidic versus physiological pH. Breast tumor cell lines were cultured at the indicated pH then assessed for sensitivity to TPT (*diamonds*), SN-38 (*circles*), MDC (*inverted triangles*), and CMMDC (*squares*). Magnitude of potentiation is defined as the IC_{50} at pH 7.4 divided by the IC_{50} at pH 6.8. Cells were exposed to agents for two cell doublings, then changed to drug-free medium for one cell doubling prior to growth inhibition assay. *Data points* Average of three independent experiments; *error bars* interassay standard error

tumors vs. 7.4 in normal tissue. Thus the average difference, or pH gradient, is approximately +0.2 pH units in normal tissue and −0.2 to −0.6 in tumor tissue. For weak acids in particular, an acidic pH_e reduces charge density, increases lipophilicity, and favors uptake across the cell membrane. Once inside the cell, the agent again becomes charged at basic pH_i and is thus trapped. Since the effect is exponential, even a small increase in the gradient can translate into a significant increase in intracellular drug accumulation.

Accordingly, alkylating agents that are weak acids are more effective in vitro and in vivo at acidic pH. Skarsgard et al. [24] reported that low pH potentiated cytotoxicity of melphalan 2-fold in human cervical squamous carcinoma cells in vitro. Similar potentiation was seen in human lung [23] and rat mammary carcinoma cell lines [7]. The carbonic anhydrase inhibitor acetazolamide, which lowers blood pH, potentiated the antitumor activity of cisplatin and melphalan in vivo [25]. Likewise, melphalan, in combination with nigericin, an ionophore that permits influx of H^+ ions to reduce pH_i, caused up to a 2-fold increase in growth delay in vivo in the RIF-1 murine tumor compared to alkylator alone [31]. Finally, lowering of tumor pH_e via a hyperglycemia-induced increase in glycolysis potentiated the activity of the bi functional alkylating agent, 1,3-bis-2-(chloroethyl-1--nitrosourea), in the BT4An rat glioma [22].

While several groups have evaluated the acute effects of pH on CPT activity in vitro [4, 25, 27], comparatively little work has been reported on pH modulation of CPTs in vivo. Gabr and coworkers [4] published preliminary animal studies in which the basal interstitial pH of murine RIF-1 tumors was selectively lowered by intraperitoneal injection of the mitochondrial inhibitor meta-iodobenzylguanidine and glucose. This method of tumor acidification had no effect on the antitumor activity of TPT but produced a 2-day increase in tumor growth delay in mice treated with CPT. These experiments likely failed to reveal the full potential of pH manipulation on CPT antitumor activity because the dose and schedule of CPT treatment and pH modulation were not optimized. However, this work serves as proof of principle, and emphasizes the need to screen for CPT analogues that possess potent antitumor activity at acidic versus physiological pH.

The traditional approach to identification of new antitumor agents has focused on in vitro screens that employ human tumor cell lines with high growth fractions cultured in monolayer at physiological pH [2]. The criteria for selection are commonly based on drug potency in either a growth inhibition or cytotoxicity assay. Chemosensitivity of tumor cells that have adapted to acidic and/or hypoxic environments is not modeled. In addition, our approach includes a post-exposure culture period in drug-free medium. This permits a more complete expression of activity for agents whose cytotoxicity mechanisms require progression through the cell cycle (e.g., paclitaxel), or protein synthesis-dependent apoptosis (doxorubicin). Conversely, post-exposure incubation allows capable cells time to repair and thus survive drug-induced damage. This could explain why we do not observe the relative potency for SN-38 that has been observed by others. For example, when the same set of analogues was tested in the NCI screen, the rank order of activity in MDA-MB-231 cells following 48 h continuous exposure at pH 7.4 was: SN-38 >> MDC = TPT > CPT = CMMDC (Ed Sausville, NCI, personal communication).

The objective of the current study was to find agents that can exploit the tumor-selective pH gradient. CPTs are good potential candidates, because at physiological

Table 2 Potentiation of established chemotherapeutic agents by acidic pH in human breast tumor cell lines antiproliferative activity of established antitumor agents from different mechanistic classes was assessed in breast tumor cell lines as described in the text. The classes represented include: topoisomerase II inhibitor (doxorubicin, *DOX*), microtubule poison (paclitaxel, *TXL*), alkylating agent (4-hydroperoxycyclophosphamide, *4-HC*), and DNA antimetabolite (5-fluorouracil, *5-FudR*). Data are the mean and standard error from three replicate experiments

Cell Line	Agent	pH 6.8 IC_{50} (nM)	Relative activity	pH 7.4 IC_{50} (nM)	Relative activity	Fold potentiation
MCF-7/wt	DOX	18 ± 3	1.0	17 ± 2	1.0	0.9
	TXL	34 ± 1	1.8	31 ± 2	1.8	0.9
	4-HC	7089 ± 430	385.9	18200 ± 1358	1074.8	2.6
	5-FUdR	12889 ± 706	701.7	15733 ± 809	929.1	1.2
MCF-7/hc	TXL	65 ± 18	1.0	45 ± 3	1.0	0.7
	DOX	108 ± 11	1.6	90 ± 13	2.0	0.8
	5-FUdR	17093 ± 2207	261.8	19557 ± 1112	434.9	1.1
	4-HC	30929 ± 5205	473.6	55567 ± 3467	235.7	1.8
MDA-MB-231	TXL	24 ± 3	1.0	14 ± 1	1.0	0.6
	DOX	140 ± 31	5.8	95 ± 4	6.8	0.7
	4-HC	7285 ± 1436	301.2	18967 ± 1235	1364.5	2.6
	5-FUdR	19316 ± 4180	798.7	15133 ± 1734	1088.7	0.8

Table 3 Effect of pH on lactone ring stability in phosphate buffer. Lactone ring stability was assessed in phosphate buffer at pH 7.4 and 6.8 by HPLC as described in the text. Results are from a representative experiment

Camptothecin analogue	Detection method	Fold increase in lactone form at pH 6.8
TPT	Fluorescence	2.1
	UV (λ_{max})	2.6
SN-38	Fluorescence	4.0
	UV (λ_{max})	3.2
MDC	Fluorescence	4.0
	UV (λ_{max})	3.5
CMMDC	UV (λ_{max})	3.0

pH they are in the hydroxy acid form that does not inhibit topoisomerase I, and that does not readily diffuse into cells. Consistent with these findings, our three human breast tumor cell lines were 5- to 65-fold more sensitive to CPT analogues at pH 6.8 than at pH 7.4. More importantly, by applying pH modulation as a selection criterion, a new rank order emerged in which the most potentiated compound, CMMDC, was not the most potent agent at physiological pH.

The precise molecular basis for pH potentiation of CPTs remains unclear. However, the redox state of the tumor cell appears to be critical. Our data indicate that intracellular GSH in particular is a cellular determinant of sensitivity to CPTs. Others have reported conflicting results on this point. For example, GSH was increased 2- to 4-fold in glioma cell lines selected in vitro for resistance to CPT-11, although total GSH-*S*-transferase and GST-π activities were unchanged [13]. While 8226/lR-5 myeloma cells, which exhibit GSH-associated multidrug resistance, showed slightly increased sensitivity to TPT, small-cell lung cancer lines with MRP-associated multidrug resistance were resistant to TPT [9]. Moreover, NYH/CIS, a cisplatin-resistant small-cell lung cancer line, expressed elevated GSH levels and was cross-

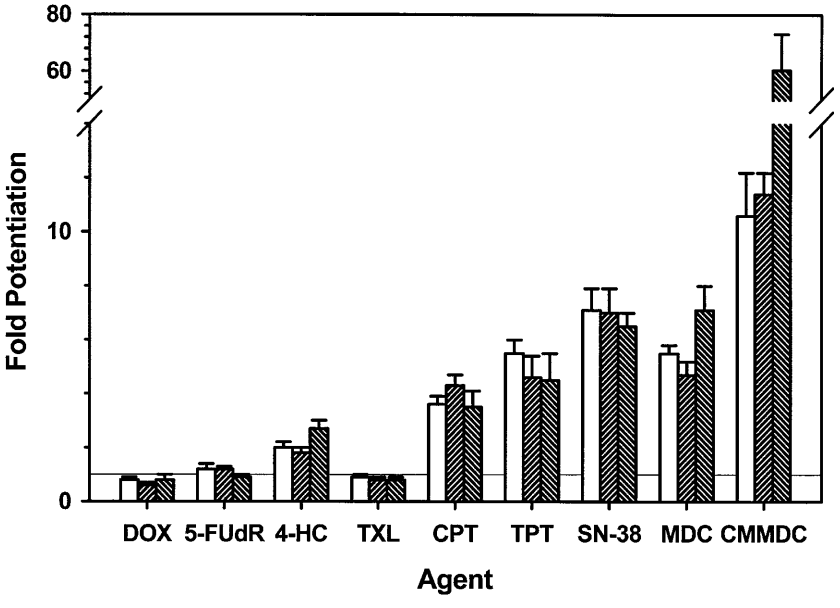


Fig. 4 Drug specificity of CPT potentiation by acidic pH. Growth inhibition assays were used to compare the activity of various classes of established antitumor agents with CPT analogues in MCF-7/wt (*open bars*), MCF-7/hc (*upward hatched bars*), and MDA-MB-231 cells (*downward hatched bars*) at pH 6.8 versus 7.4. Results represent the average of three independent experiments; *error bars* interassay standard error

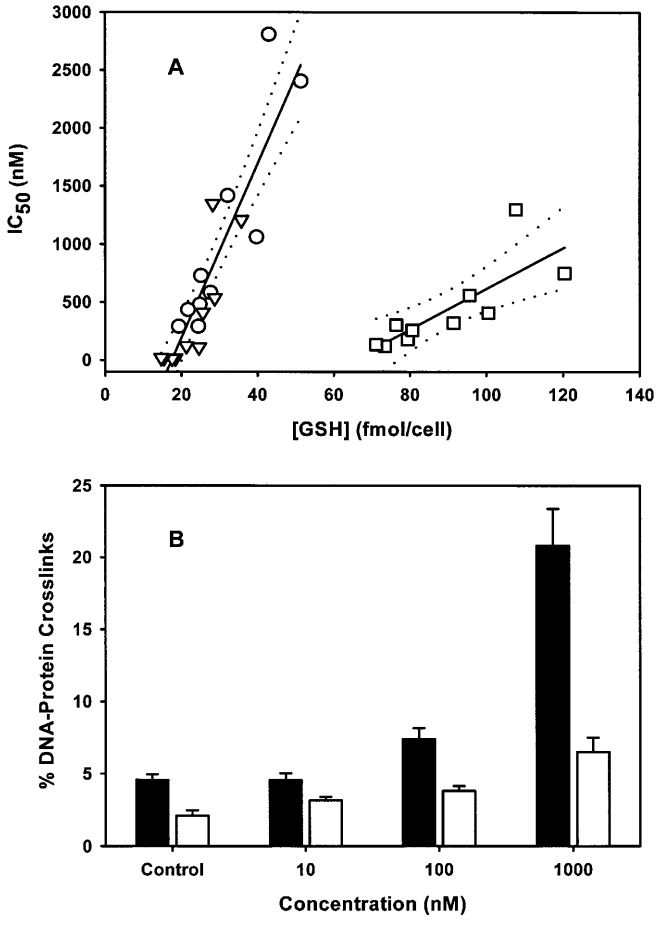


Fig. 5A,B Potential mechanisms underlying pH potentiation of CMMDC. **A** Correlation of antiproliferative activity of CMMDC with intracellular GSH. Human breast cancer cell lines were adapted to growth at pH 6.6, 6.8, 7.0, 7.2, and 7.4 and then evaluated for sensitivity to CMMDC, and for intracellular GSH as described in the text. The figure combines data for MCF-7/wt (*circles*), MCF-7/hc (*squares*), and MDA-MB-231 (*triangles*) cells from two separate experiments. **B** Effect of pH on CMMDC-induced DNA protein crosslinks. MCF-7/hc cells were labeled with [^{3}H]thymidine, exposed to control medium (*open bars*) or medium containing CMMDC (*filled bars*) for 24 h. Cells were washed briefly, lysed, and DNA-protein crosslinks quantified by the potassium SDS precipitation assay

resistant to CPT-11 and TPT [8]. Our results with MCF-7/hc cells indicate that elevated levels of intracellular GSH alone versus that in MCF-7/wt are not correlated with sensitivity to CPTs, which are slightly more active in the 4-HC resistant cell line. This suggests that the relationship of CPT sensitivity to GSH metabolism across cell lines is more complex. It is conceivable that parameters such as GSH turnover rate or GSH utilization by MRP, glutaredoxin, or thioredoxin play a key role in cellular sensitivity to CPTs. If so, perturbation of GSH pathways should affect CPT activity. Our preliminary results indicate that antiproliferative activity of CMMDC is increased approximately 12-fold by prior treatment of MDA-MB-231 cells with either buthionine sulfoximine or ethacrynic acid, agents that deplete intracellular GSH [1]. However, buthionine sulfoximine did not alter sensitivity to SN-38 in HAC2 ovarian carcinoma cells or in a cisplatin-resistant variant [17].

In summary, screening compounds against human breast tumor cells adapted to growth at acidic pH has revealed CPT analogues that could exploit the pH gradient unique to human tumors. The level of potentiation by pH differs among cell lines, suggesting a complex mechanism. One possible determinant for such selectivity is the cellular redox state, which is characterized by reduced levels of GSH that could increase the efficiency of CPT-induced DNA damage.

Acknowledgements This work was supported by NIH grant UO1 CA68697-02.

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