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Camptothecin analogs with enhanced activity against human breast cancer cells. II. Impact of the tumor pH gradient

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Abstract Human breast tumors often exist in an acidic and hypoxic microenvironment, which can promote resistance to radiation and chemotherapies. A tumor-selective pH gradient arises in these tumors which favors uptake and retention of drugs like camptothecin that are weak acids. We evaluated the effect of alkyl substitutions at the 7 position in seven CPTs with varying groups at the 10 position on modulation by acidic extracellular pH in three human breast cancer cell lines. Growth inhibition was assessed by propidium iodide staining of nucleic acids in human breast cancer cells cultured at either extracellular pH 6.8 or 7.4 that were (1) hormone-sensitive (MCF-7/wt), (2) hormone insensitive (MDA-MB-231), or (3) alkylator-resistant (MCF-7/4-hc). Over 10-fold pH modulation was observed in 7-halomethyl analogs of methylenedioxy-CPT and in 7-alkyl analogs of 10-amino-CPT. Of 39 analogs tested, the overall pattern of activity across breast tumor cell lines was similar with some notable exceptions. For example, 7-propyl-10-amino-CPT was modulated 16- to 20-fold by acidic extracellular pH in the MCF-7 cell lines, but only 6-fold in MDA-MB-231 cells. One mechanism that can contribute to pH modulation is enhanced cellular drug uptake and retention. In MCF-7/wt cells, uptake of 10-amino-CPT increased 4-fold, while retention increased over 10-fold at acidic extracellular pH. In addition, gene expression analysis of MCF-7/wt cells indicated that expression of a number of genes changed under acidic culture conditions, including down-regulation of the CPT efflux protein pump breast cancer resistance protein (BCRP). Interestingly, expression of topoisomerase I, the molecular target of CPT, was not affected by acidic growth conditions. These results highlight the importance of maintaining key features of tumor physiology in cell culture models used to study cancer biology and to discover and develop new anticancer drugs. While several substitutions at the 7 and 10 positions enhance potency, 7-halomethyl and 10-amino CPT analogs show selective activity at the acidic pH common to the microenvironment of most solid tumors.

Keywords Camptothecin · pH gradient · Breast cancer Drug development · Microarray

Abbreviations top1: Topoisomerase I · CPT: Camptothecin · MD or MDO: 10,11-methylenedioxy · ED: 10,11-ethylenedioxy · MeO: Methoxy · DFMD: Difluoromethylene dioxy · CMMDC: 7-chloromethyl-10,11-methylenedioxy-camptothecin · SN-38: 7-ethyl-10-hydroxy-camptothecin

The work is dedicated to the memory of Dr. Monroe E. Wall, who inspired this research team and many other investigators committed to creating useful anticancer drugs from natural products.

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Introduction

Sequencing of the human genome gave rise to the genomic era of cancer research that has created a dramatic shift from traditional, largely empirical drug discovery and development to a mechanism-based

model focused on signal transduction pathways. This current focus on the molecular level of organization and interaction often overshadows consideration of higher levels of organization and complexity. For example, there is substantial literature dating to Otto Warburg in the 1930's, which documents that solid tumors commonly exist in an acidic and hypoxic microenvironment due to defects in regulation of glycolysis and inefficient tumor vasculature. Paradoxically, this tumor-selective microenvironment has not been routinely incorporated into models for anticancer drug development. Indeed, most of our expanding knowledge of tumor biology, including that for signal transduction pathways, has been derived largely from cells cultured in two dimensions under optimal growth conditions. Patterns of gene expression can change significantly under adverse growth environments [16, 36]. A notable example is the transcription factor hypoxia-inducible factor 1α (HIF-1α). Under hypoxic conditions, degradation of HIF-1α is decreased, which leads to elevation of HIF-1α and subsequently to changes in transcription of over 60 genes. Of these, 11 are glycolytic enzymes whose activity increases dramatically along with glucose consumption to create the tumor metabolic phenotype [30]. In addition, an acidic microenvironment can significantly impact drugs that are weak acids or weak bases, since charged molecules do not readily cross cell membranes. Moreover, tumor cells maintain a neutral to alkaline intracellular pH which creates a tumor-selective pH gradient [8]. Drugs that are weak acids will thus be protonated in this environment, and will passively diffuse across cell membranes where they will again become charged and thus trapped at the higher intracellular pH. This ion trapping effect can lead to a considerable difference in cellular drug accumulation in tumor cells in culture [18, 26] and in spontaneous canine tumors in vivo [24].

A number of second generation CPT analogs are now in clinical development (reviewed in [42]). Improved blood stability, solubility and activity against CPT-resistant cell lines are the primary new attributes of these analogs. Engineering greater tumor selectivity into the molecule has received relatively less attention. Instead new CPT analogs have relied for selectivity on the fact that certain human tumors tend to over-express top1 compared to normal tissues [9, 11, 13, 23, 32]. We have recently reported that certain CPT analogs are significantly more cytotoxic in tumor cells cultured in vitro at acidic pH [1], which more closely resembles tumor biology observed in vivo [31, 39]. This effect is perhaps not surprising, since camptothecin contains a labile E-ring lactone that opens to form the inactive hydroxyacid at physiological pH, but closes to generate the active species at acidic pH. Thus, the analogs would be more stable in acidic medium (pH 6.8). However, the observed pH modulation (up to 40-fold) was well in excess of the 4-fold difference that could be accounted for by E-ring dynamics alone. This result suggests that certain CPT analogs would not only re-activate in the tumor microenvironment, but could also exploit the tumor-selective pH gradient to concentrate in tumor cells [8]. We were therefore interested in evaluating modifications of camptothecin that would not only improve potency in general, but also result in modulation by acidic pH. Our results indicate that seven substitutions at C-10 as well as increasing lipophilicity at C-7 enhance potency in vitro. Analogs in the 10-amino and 7-halomethyl series showed 8- to 40-fold increases in activity at acidic pH, which could provide a new approach to enhancing selectivity for camptothecin-based chemotherapeutics.

Materials and methods

Syntheses of the 7-alkyl CPT analogs used in this study are detailed in the companion paper as are methods for the growth inhibition assay and conditions for culture of human breast cancer cell lines. To adapt tumor cells to an acidic environment, the pH of the culture media was lowered to 6.8 by lowering the concentration of sodium bicarbonate, and supplementing with sodium chloride to maintain equivalent osmolarity. Cell lines were passaged at least twice in low pH medium before assessment of chemosensitivity. All incubations were at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Measurement of intracellular pH

Intracellular pH was measured with C. Snarf-1-AM (Molecular Probes, Eugene, OR) using the method described by Wahl and colleagues [37, 38]. Five spectra were acquired every 10 s from four separate fields of dye-loaded cells cultured on tumor stromal proteins (Matrigel, Collaborative Biomedical Products, Bedford, MA) in triplicate microwell dishes at extracellular pH 7.3 or 6.7. The mean and standard error were then computed over all measurements ($n = 60$).

Preparation of RNA samples and microarray analysis

Total RNA was extracted from confluent MCF-7 cells grown at pH 7.4 or adapted to growth at pH 6.8 by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Ten micrograms of total RNA was used in the first strand cDNA synthesis using T7-$(dT)_{24}$primer (GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCG $G(dT)_{24}$) (Operon Technologies, Alameda, CA) and Superscript II (Invitrogen Life Technologies, Carlsbad, CA). The second strand synthesis was carried out at 16°C in the presence of *E. coli* DNA ligase, *E. coli* DNA polymerase and *E. coli* RNase H. The newly synthesized cDNA was blunt ended with T4 DNA polymerase. Cleanup of the double stranded cDNA was carried out by phenol/chloroform extraction and ethanol precipitation using the phase lock gel system (Brinkman Instru-

ments, Westbury, NY). In order to obtain biotin labeled cRNA, an in vitro transcription reaction of the purified cDNA was performed using the BioArray High yield RNA transcript Labeling Kit (Affymetrix Inc, Santa Clara, CA). Following purification of the cRNA using RNeasy columns (QIAGEN, Valencia, CA), the cRNA was fragmented by incubating in a buffer containing 200 mM tris-acetate (pH 8.1), 500 mM KOAc and 150 mM MgOAc at 94°C for 35 min. The fragmented cRNA was hybridized with a pre-equilibrated Affymetrix chip for 16 h at 45°C. Post hybridization, the array was washed with a non-stringent buffer (6x SSPE, 0.01% Tween 20) followed by a high stringency wash in 100 mM MES, 0.1 M NaCl and 0.01% Tween 20. Following the washings, the array was stained with strepto-avidin phycoerythrin and then incubated with biotinylated goat anti-avidin antibody and restaining with SAPE. The washings and staining were performed in a GeneChip Fluidics Station 400. The chips were scanned in a HP ChipScanner (Affymetrix) to detect hybridization signals.

Data analysis

For each experimental condition i.e. pH 7.4 and 6.8, the hybridizations were performed in triplicate. Hybridization data from text files were imported into a Microsoft Excel spreadsheet. Affymetrix Microarray Suite 5.0 was used for image analysis and GeneSpring was used for data analysis. Statistically significant genes were identified by a three step filtering process: (a) genes had to be present in all samples, (b) one way ANOVA with $P < 0.05$, and (c) twofold up- or down-regulated in treatment (pH 6.8) versus controls (pH 7.4). Applying these filters, we obtained a list of 38 differentially expressed genes.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Validation of gene expression was done by RT-PCR, which was performed on total RNA prepared by TRIzol reagent (Invitrogen). Following DNase I treatment, RNA was reverse transcribed with random decamers using the RETROscript kit (Ambion, Austin, TX). The reaction mix (20 µl) contained 2 µg of total RNA, 5 µm random decamers, 500 µm dNTP, 10 U of RNAse inhibitor and 100 U of MMLV-RTase. RNA was reverse transcribed for 1 h at 42°C. PCR was performed with 1 µl of cDNA template. DNA was amplified by an initial incubation at 94°C for 5 min followed by 30 cycles of 94°C for 0.5 min, 58°C for 0.5 min, 72°C for 0.5 min, and a final extension at 72°C for 5 min. To obtain a semi-quantitative result, Quantum RNA 18S (Ambion) was used as an internal standard. The number of PCR cycles was optimized in each case to ensure that the product intensity fell within the linear phase of amplification. Unique oligonucleotide primer pairs were designed using software PRIMER3 (available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and using the sequence data from the National Center for Biotechnology Information data base. Gene-specific primers were synthesized by the Duke DNA synthesis facility. The signal intensity of the RT-PCR products was measured using Kodak 1D Image analysis software .

Results

Structures of camptothecin analogs

Work from several laboratories has indicated that the 7 position in camptothecin is available for substitutions that modulate activity (reviewed in [17, 22]). Increasing lipophilicity at this position produces analogs with greater potency and stability in human plasma [2, 19, 33]. Similarly, certain substitutions at the 10 position increase potency of CPT [14, 15, 22, 35]. How these design modifications affect pH modulation and thus selectivity of CPT analogs for solid tumors is not known. Therefore, we examined a number of 10-substituted CPT's for the effect of increasing lipophilicity at the 7 position on both potency and modulation by acidic extracellular pH. The structures of CPT analogs in clinical use and the pH-dependent equilibrium between open and closed forms of the E ring lactone are shown in Fig. 1.

Acidification of extracellular pH creates a pH gradient in human breast cancer cells in vitro

To determine the impact of adaptation to acidic extracellular pH on intracellular pH, we loaded cells from each breast tumor cell line with the fluorescent indicator dye, C. Snarf-1-AM, which exhibits spectral shifts as a function of pH upon cleavage to the free acid form (C. Snarf-1) by intracellular esterases. The results shown in Table 1 indicate that all three tumor cell lines maintain a neutral intracellular pH in the face of an acidic extracellular pH. The intracellular/extracellular pH gradient was greatest in the MCF-7 lines (−0.2 pH units), consistent with the data of others [25]. Furthermore, addition of up to 10 µM SN-38 had no effect on either extracellular pH or intracellular pH (data not shown).

Substitutions at the 10 position increase potency of camptothecin against human breast cancer cell lines at acidic extracellular pH

The effects of substitutions at the 10 position on camptothecin were evaluated in human breast cancer cell lines that represent hormone-sensitive (MCF-7/wt), hormone-insensitive (MDA-MB-231), and alkylator-

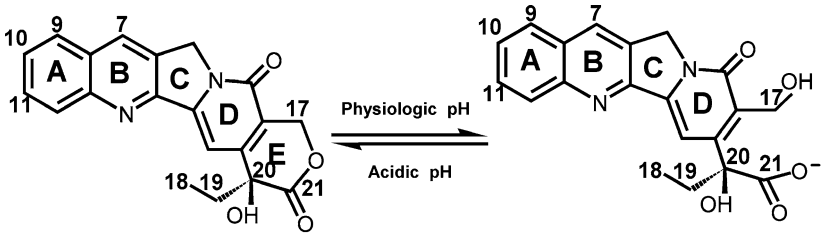
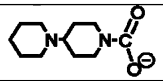


Fig. 1 Structures of camptothecin analogs. Structures are shown for the CPT analogs approved for clinical use. At physiological pH, the E-ring lactone of CPT will open to form the inactive hydroxyacid. This process is reversible at acidic pH

Analog	C5	C7	C9	C10	C20 (S)
CPT	H	H	H	H	OH
TPT	H	H	$(CH_3)_2NHCH_2$	OH	OH
CPT-11	H	C_2H_5	H		OH
SN-38	H	C_2H_5	H	OH	OH

resistant forms of the disease (MCF-7/hc). The results shown in Table 2 confirm that this is an activating position, since potency increased on average for the three cell lines from 19 (10-OH) to 43-fold (10,11-MD) compared to CPT itself. The 10,11-methylenedioxy- and 10,11-ethylenedioxy-CPT analogs were the most potent with the rank order of activities being: 10,11-MD≥10,11-ED > 10,11-DFMD > MD > 10-OMe = 10-NH2 > 10-OH≫CPT. On average over the three cell lines, pH modulation was 3 to 4-fold for each analog (Fig. 2), which is likely due to stabilization of the E-ring lactone at acidic pH [1]. However, there was one notable exception, 10-amino-CPT, in which the average modulation exceeded 8-fold, over twice that observed for CPT or for SN-38, the active metabolite of the clinically established top1 inhibitor, irinotecan. While the general pattern of pH modulation was similar among cell lines, there were some differences. For example, 7-propyl-10-amino-CPT was modulated 16- to 20-fold in the MCF-7 cell lines, but only 6-fold in the MDA-MB-231 line (Fig. 2). On the other hand, CMMDC was modulated 23-fold in MDA-MB-231, 37-fold in MCF-7/wt, but only 6-fold in MCF-7/hc cells, which express elevated levels of glutathione [1] that can react with alkylating moieties like the 7-chloromethyl group.

Increasing alkyl chain length produces enhanced potency in each 10-substituted CPT analog at acidic extracellular pH

Increasing the carbon chain length and thus lipophilicity at the 7 position in each 10-substituted CPT analog lead to an increase in potency compared to the unsubstituted analog (Table 2). On average, there was a linear increase with chain length, producing a maximum 13-fold increase in activity for CPT itself, and a 2.5 to 5.5 increase for the 10-substituted analogs. Hence, increasing lipophilicity at the 7 position had a positive effect on potency, but it was minor compared to that conferred by modifications at the 10 position.

Table 1 Effect of extracellular pH (pH_e) on intracellular pH (pH_i)

Cell line	Measured pH_i at specified pH_e		pH gradient (pH_e–pH_i)	
	pH_e 7.4	pH_e 6.8	pH_e 7.4	pH_e 6.8
MDA-MB-231	7.54 ± 0.04	6.88 ± 0.06	−0.14	−0.08
MCF-7/wt	7.42 ± 0.08	7.00 ± 0.01	−0.02	−0.20
MCF-7/hc	7.29 ± 0.03	6.99 ± 0.02	0.11	−0.19

7-halomethyl substituents reduce potency but increase modulation by acidic pH

Addition of a 7-halomethyl group on 10-substituted CPT's decreases potency at pH 7.4 on average by 10 (10,11-DFMD) to 37 (10-OH)-fold in the breast cancer cell lines, with the effect somewhat more prominent for bromine versus chlorine analogs (see companion paper). Likewise, at acidic extracellular pH potency decreased for all 10-substituted analogs from 2 (10,11-DFMD) to 17-fold (10-OH; Table 3). However, this decrease was still less than that observed at extracellular pH 7.4, and one of these analogs, 7-chloromethyl-10,11-MD-CPT, displayed the greatest pH modulation (40-fold in MCF-7/wt), almost twice that for the corresponding CPT analog (Fig. 2), and in good agreement with our previous results that employed a different growth inhibition assay that utilized a metabolic endpoint [1] (Fig. 3).

Ranking compounds by potency differs from that by pH modulation

Next, we examined the agreement between selection of compounds based on potency at physiological pH compared to selection based on pH modulation (IC_{50} at physiological extracellular pH/IC_{50} at acidic extracellular pH). Figure 4 shows results for the ranking of all 39 analogs in each cell line. The data are widely dispersed about the ideal correlation line, with linear regression correlation coefficients less than 0.5. Taking another approach, we determined which analogs were ranked among the top six agents by both selection methods. By

Table 2 Antiproliferative activity of 10-substituted and 7-alkyl-substituted CPT analogs against human breast cancer cells adapted to growth at acidic pH in vitro

Ring system	7-Alkyl	IC_{50} (nM)					
		MDA-MB-231	Fold[a]	MCF-7/wt	Fold[a]	MCF-7/hc	Fold[a]
CPT	(none)	591 ± 11	1	151 ± 18	1	257 ± 13	1
	Methyl	104 ± 3	6	32 ± 3	5	39 ± 6	7
	Ethyl	84 ± 5	7	22 ± 4	7	31 ± 5	8
	Propyl	79 ± 3	7	13 ± 2	12	35 ± 3	7
	Butyl	43 ± 4	14	11 ± 3	14	21 ± 1	12
10,11-MD	(none)	11 ± 1	54	4 ± 0	38	7 ± 0	37
	Methyl	6 ± 0	99	4 ± 1	38	3 ± 0	86
	Ethyl	4 ± 1	148	3 ± 1	50	2 ± 0	129
	Propyl	2 ± 1	296	1 ± 0	151	1 ± 0	257
	Butyl	2 ± 0	296	1 ± 0	151	1 ± 0	257
10-OH (SN-38)	(none)	38 ± 6	16	8 ± 2	19	12 ± 2	21
	Methyl	29 ± 10	20	5 ± 0	30	11 ± 3	23
	Ethyl	19 ± 4	31	4 ± 1	38	7 ± 1	37
	Propyl	18 ± 4	33	3 ± 0	50	6 ± 1	43
	Butyl	19 ± 3	31	2 ± 0	76	5 ± 2	51
10,11-ED	(none)	13 ± 2	45	6 ± 1	25	5 ± 1	51
	Methyl	9 ± 1	66	5 ± 1	30	4 ± 0	64
	Ethyl	4 ± 0	148	3 ± 1	50	4 ± 0	64
	Propyl	3 ± 1	197	3 ± 0	50	2 ± 0	129
	Butyl	3 ± 0	197	3 ± 0	50	1 ± 0	257
10-NH2	(none)	51 ± 4	12	14 ± 1	11	5 ± 0	51
	Methyl	43 ± 5	14	11 ± 1	14	7 ± 0	37
	Ethyl	31 ± 4	19	12 ± 2	13	6 ± 0	43
	Propyl	19 ± 4	31	5 ± 0	30	2 ± 0	129
	Butyl	8 ± 1	74	4 ± 0	38	2 ± 0	129
10-OMe	(none)	21 ± 2	28	15 ± 3	10	7 ± 1	37
	Methyl	15 ± 1	39	10 ± 2	15	6 ± 1	43
	Ethyl	11 ± 2	54	7 ± 0	22	6 ± 2	43
	Propyl	9 ± 1	66	5 ± 1	30	5 ± 1	51
	Butyl	8 ± 1	74	3 ± 1	50	4 ± 0	64
10,11-DFMD	(none)	21 ± 1	28	4 ± 0	38	6 ± 1	43
	Ethyl	32 ± 4	18	5 ± 1	30	8 ± 2	32

[a] Fold increase in growth inhibitory activity versus CPT defined as $IC50_{CPT}/IC50_{analog}$

chance alone, even if there were no agreement between the selection methods, we would expect one drug to be selected by both methods that is among the top six. However, within each of the three forms of the disease, no analogs were ranked among the top six agents by both selection methods. These data suggest that potency and pH modulation can be independent criteria for discovery of new CPT-based antitumor agents.

Mechanisms of pH modulation

Cellular uptake and retention

To determine whether ion trapping was a mechanism for pH modulation observed for 7-butyl-10-amino CPT (BACPT), MCF-7/wt cells were exposed to the analog to assess uptake and efflux. This analog has the advantageous property of being highly fluorescent, and thus uptake, efflux and retention can be evaluated in both live cells and in extracts by fluorescence spectroscopy. The results shown in Fig. 4 indicate that cellular uptake saturates within an hour at either extracellular pH 7.4 or 6.8. However, cells cultured at acidic extracellular pH accumulated about four times the drug compared to those cultured at physiological extracellular pH. More important, efflux was rapid, with most BACPT eliminated within 20 min. However, cells cultured at acidic extracellular pH retained approximately 40 times the amount in control cells when switched to drug-free medium, suggesting that ion trapping had occurred. This difference in drug retention alone could account for the pH modulation observed for BACPT in MCF-7/wt cells.

Changes in gene expression with adaptation to acidic culture conditions

When MCF-7/wt cells were analyzed by gene array, distinct patterns of overall gene expression were observed between culture at physiological versus acidic extracellular pH. GeneSpring analysis of the dataset identified 38 transcripts with statistically significant differences in their expression pattern. Twelve transcripts were found to be up-regulated (Table 4) and 28 down-regulated (Table 5) on exposure to acidic conditions, including genes involved in signal transduction (FZD2), stress response (MICB), tumor suppression (BNIP3L), cell proliferation (GAS6) and metabolism (ALDH9A1, BLVRB), drug resistance (SEMA3C), drug binding (SR-BP1) and DNA repair (ADPRTL2). These gene functions could play a role in drug uptake and response. Expression data for three randomly chosen genes, namely Carbonic anhydrase IX (CA9), Growth arrest specific-6(GAS6) and BNIP3L were validated by semi-

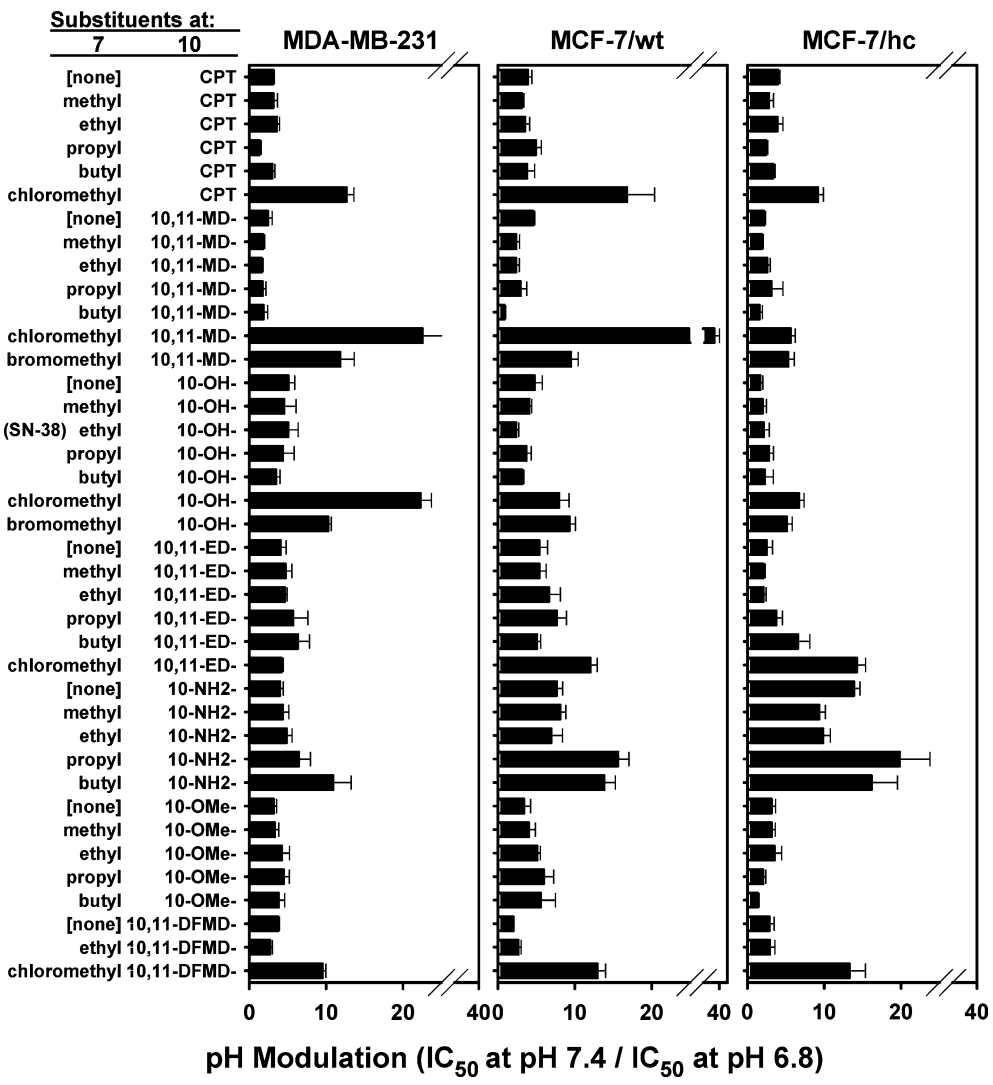


Fig. 2 pH modulation of CPT analogs in three human breast tumor cell lines. CPT analogs were evaluated by growth inhibition assay in three human breast tumor cell lines grown at either pH 7.4 or pH 6.8. The IC_{50} was determined from dose response curves and used to compute the fold pH modulation, defined as the ratio of the IC_{50} at pH 7.4 to that at pH 6.8. *Error bars* represent the inter-experimental variation from 3 to 5 replicate assays

quantitative RT-PCR. The microarray and the RT-PCR data were in close agreement with each other. No change in expression for CA9 was detected by either microarray or RT-PCR. GAS6 showed a 2.8-fold induction by microarray and a 2.2-fold induction by RT-PCR. BNIP3L showed a 2.1-fold decrease in expression by micorarray analysis and a 1.6-fold repression in expression by RT-PCR. A decrease in the expression of the CPT efflux pump, BCRP under acidic conditions (Fig. 5) was observed by RT-PCR (a probeset corresponding to BCRP was not present on the array). The down-regulation of SEMA3C and BCRP and the up-regulation of SR-BP1 under acidic conditions could affect the sensitivity of MCF-7 cells to CPT's via increased intracellular drug accumulation as observed above. Of note, the expression levels of topoisomerase I remained unchanged and an up-regulation in a DNA repair gene was observed on exposure to acidic conditions. These observations are consistent with a previous study where no change in topoisomerase I activity was observed on exposure of 55 cell lines to topoisomerase I inhibitors [5].

Table 3 Antiproliferative activity of 7-halomethyl-substituted CPT analogs against human breast cancer cells adapted to growth at acidic pH in vitro

Ring system	7-halogen	IC_{50} (nM) at pH 6.8		
		MDA-MB-231	MCF-7/wt	MCF-7/hc
CPT	Chloro	123 ± 8	39 ± 8	101 ± 7
10,11-MD	Chloro	24 ± 4	13 ± 1	40 ± 4
10,11-MD	Bromo	59 ± 9	62 ± 4	51 ± 7
10-OH	Chloro	157 ± 7	159 ± 14	228 ± 13
10-OH	Bromo	406 ± 12	204 ± 10	271 ± 24
10,11-ED	Chloro	184 ± 9	57 ± 4	34 ± 2
10,11-DFMD	Chloro	80 ± 2	7 ± 0	13 ± 2

Discussion

The majority of solid tumors including breast cancer contain regions of acute/chronic hypoxia due to abnormal tumor vasculature and the associated inadequate blood supply (reviewed in [40, 41]). Tumor hypoxia is associated with increased malignancy and poor prog-

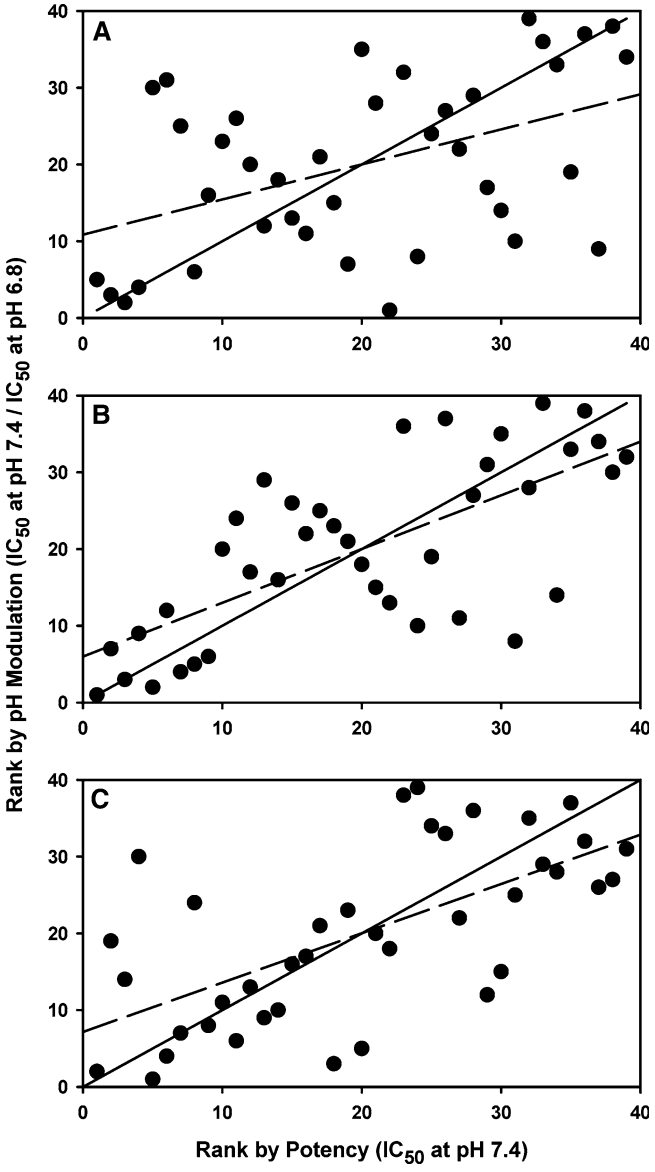


Fig. 3 Correlation of potency to pH modulation in human breast cancer cells. Potency, defined as the IC_{50} for CPT analogs was plotted against pH modulation, defined as the ratio of the IC_{50} at pH 7.4 to that at pH 6.8, for MDA-MB-231 (**a**), MCF-7/wt (**b**), and MCF-7/hc (**c**) breast cancer cell lines ($n = 39$). The linear regression (*dashed*) lines had slopes significantly different from the slope of the ideal correlation (*solid*) line. Linear correlation coefficients were 0.21, 0.46 and 0.41, with slopes of 0.46 ± 0.15, 0.70 ± 0.12, and 0.64 ± 0.13, respectively

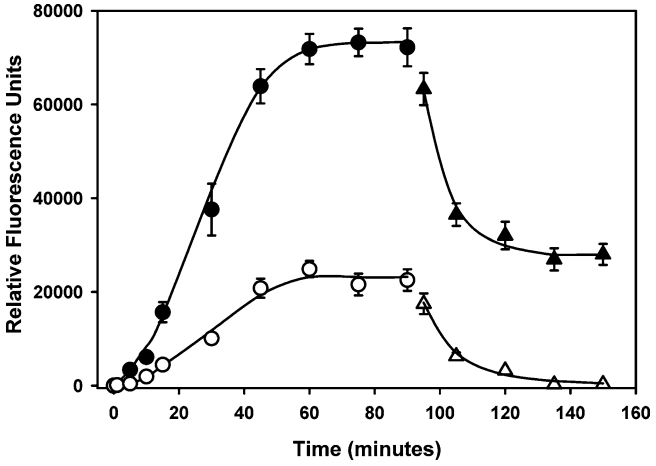


Fig. 4 Uptake and efflux of BACPT as a function of extracellular pH. MCF-7 human breast cancer cells (10^6/T25 flask) were cultured at either pH 7.4 (*open symbols*) or pH 6.8 (*closed symbols*) and exposed to 50 μM BACPT for the indicated times. Cells were then extracted with 0.5N perchloric acid, centrifuged, neutralized with KOH, centrifuged to remove potassium perchlorate, and the supernatant was re-acidified and then analyzed for uptake of BACPT on a fluorescence microplate reader (*circles*). For drug retention, cells were exposed to 50 μM BACPT for 1.5 h, then switched to drug-free medium for the indicated times prior to extraction and analysis for BACPT (*triangles*)

nosis, due in part to a higher resistance to radiation therapy and certain chemotherapies that require oxygen or cell cycle progression in their mechanism of cell cytotoxicity [4, 41]. Indeed, 30–40% of breast carcinomas have pO_2 values less than 10 mm Hg, compared to 54–65 mm Hg for normal tissue [12, 27, 34]. Hypoxia leads to induction of the transcription factor hypoxia-inducible factor 1 (HIF-1), which is expressed in the majority of patients with node-positive breast cancer and has been proposed as an independent predictor for unfavorable prognosis [3, 10, 29]. Of the over sixty genes regulated by HIF-1, thirteen up-regulate either glycolysis or glucose transport and hence HIF-1 links hypoxia with acidosis [30]. Accordingly, the extracellular pH of breast tumors can be as low as 6.48 [39], while the intracellular pH is closely maintained at around 7.15 [8]. These results indicate that a pH gradient likely exists in the majority of breast tumors, and especially those that have progressed. Thus, there is a sound rationale for developing drugs that can exploit this tumor-selective pH gradient.

Clinical success of the camptothecin analogs, topotecan and irinotecan has validated top1 as a molecular target for cancer chemotherapy. However, issues of potency and dose limiting toxicity have spurred investigations aimed at creating the next generation of CPT-based anticancer drugs. Considering the growing importance of tumor physiology on drug and radiation response, we have sought to identify CPT analogs that can exploit the pH gradient known to exist in solid tumors and not in normal cells [8, 37, 38]. The camptothecins are particularly well-suited to this approach, because they contain a labile E ring lactone that forms an inactive, membrane impermeable hydroxy-acid at physiological pH, while the active species will predominate at the acidic pH typical of the tumor microenvironment. Our strategy was to identify CPT analogs with potent and selective activity at acidic pH that can then be formulated as highly water-soluble 20(S) dipeptide pro-drugs where the E-ring lactone is protected from conversion to the hydroxy acid and subsequent binding to human serum albumin. We have previously reported discovery of such analogs [1], and sought to extend this approach to other CPT's modified at both the C-10 and

Table 4 Gene transcripts up-regulated in MCF-7/wt cells at acidic extracellular pH

Gene	Function	Normalized intensity		Fold change
		pH 7.4	pH 6.8	
SRBP1	Drug binding, receptor	0.27	1.39	5.2
SNL	Cell shape, proliferation	0.50	2.02	4.0
MICB	Cellular defense	0.53	1.55	2.9
GAS6	Signal transduction	0.61	1.70	2.7
FZD2	Receptor	0.51	1.41	2.7
PACE4	Cell signalling	0.57	1.49	2.6
TXNIP	Thioredoxin interacting protein	0.55	1.37	2.5
CRA	Cisplatin resistance associated protein	0.61	1.53	2.5
TCF8	Transcription factor	0.69	1.65	2.4
ADPRTL2	DNA repair	0.64	1.42	2.2
EST	Unknown	0.60	1.40	2.2
CNP	Diesterase	0.66	1.44	2.2

C-7 positions. We discovered two modifications that produce analogs with significantly greater antiproliferative activity at acidic pH: the alkylating halomethyl substitution at C-7, and the amino substitution at C-10. These effects are not simply artifacts of monolayer cell culture or specific for breast cancer cells, since we have made similar observations with several other tumor types in 3-D histoculture [6]. The precise mechanism(s) of pH modulation affected by these modifications are not known, but several may be involved. First, our initial studies indicate that breast tumor cells adapted to growth at acidic pH have a decreased level of glutathione (GSH) that closely correlates with increased sensitivity to CPT's [1]. Several groups have also found that reduction of intracellular GSH synthesis by treatment with buthionine sulfoximine sensitizes cells to CPT's, which supports a central role for GSH and GSH metabolism in the cytotoxicity of camptothecins [7, 20, 21, 28]. Second, enhanced uptake and retention due to the tumor pH gradient can clearly affect the activity of analogs like 7-butyl-10-NH_2-CPT. Third, DNA microarray analyses on MCF-7/wt cells cultured at acidic versus physiological pH reveal changes in expression of genes involved in transport, cell–cell communication, metabolism, and apoptosis; pathways that could likely impact drug response. In summary, these studies on 7-

Table 5 Gene transcripts down-regulated in MCF-7/wt cells at acidic extracellular pH

Gene	Function	Normalized intensity		Fold change
		pH 7.4	pH 6.8	
CXCR4	Receptor	1.65	0.49	−3.3
RPA3	DNA replication	1.74	0.56	−3.1
RPA3	DNA replication	1.57	0.61	−2.6
KDELR3	Receptor	1.45	0.56	−2.6
ALH9A1	CHO metabolism	1.41	0.58	−2.4
SEMA3C	Drug resistance	1.46	0.61	−2.4
DDT	Isomerase	1.36	0.58	−2.3
BLVRB	Metabolism	1.53	0.66	−2.3
TOM1L1	Transport	1.53	0.66	−2.3
CEBPG	Transcription factor	1.40	0.60	−2.3
ECSH1	Fatty acid metabolism	1.45	0.65	−2.2
AP2S1	Secretory protein	1.26	0.56	−2.2
TP1	CHO metabolism	1.36	0.61	−2.2
C12ORF8	Intracellular protein	1.36	0.62	−2.2
MLH1	DNA repair	1.29	0.59	−2.1
DDT	Isomerase	1.39	0.64	−2.1
BNIP3L	Tumor suppressor	1.30	0.61	−2.1
SORD	CHO metabolism	1.39	0.65	−2.1
PPP1CC	Phosphatase	1.47	0.69	−2.1
C4.4A	Metastasis	1.31	0.62	−2.1
ME1	CHO metabolism, electron transporter	1.39	0.66	−2.0
PSMA2	Proteasome	1.39	0.68	−2.0
NSF	CHO metabolism	1.47	0.72	−2.0
DKFZP566	Unknown	1.29	0.64	−2.0
PAM	Electron transporter	1.35	0.67	−2.0
PDE8A	Diesterase	1.28	0.64	−2.0
LOC927	Unknown	1.31	0.65	−2.0

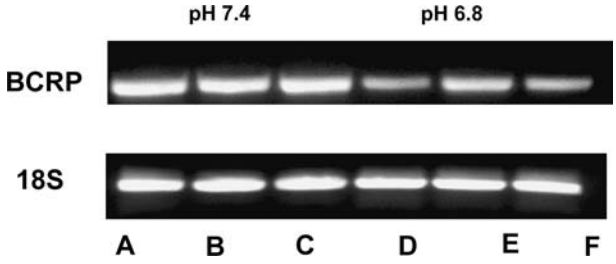


Fig. 5 Down-regulation of BCRP at acidic extracellular pH. Relative mRNA levels of BCRP (Breast cancer resistance protein) in MCF-7 cells cultured at physiological and acidic pH were determined by semi-quantitative RT-PCR. Lanes 1,2 and 3 depict replicates of cells cultured at pH 7.4 and lanes 4,5, and 6 depict replicates of samples cultured at pH 6.8. 18S ribosomal RNA was used as the internal control to determine the relative amount of BCRP in each sample. Representative photograph of the agarose gel for the RT-PCR product stained with ethidium bromide is displayed

and 10-substituted camptothecins suggest new avenues for synthesis of analogs that could exhibit improved tumor selectivity while retaining high antitumor activity, and confirm our earlier observation that selection of compounds based on pH modulation will generate a distinctly different rank order than that based on potency alone.

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