Design, Synthesis, and Evaluation of pH-Dependent Hydrolyzable Emetine Analogues as Treatment for Prostate Cancer

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Ⓢ *Supporting Information*

ABSTRACT: The N-2′ position of the natural product emetine has been derivatized to thiourea, urea, sulfonamide, dithiocarbamate, carbamate, and pH responsive hydrolyzable amide analogues. In vitro studies of these analogues in PC3 and LNCaP prostate cancer cell lines showed that the analogues are generally less cytotoxic (average IC_{50} ranging from 0.079 to 10 μM) than emetine (IC_{50} ranging from 0.0237 to 0.0329 μM). The pH sensitive sodium dithiocarbamate salt **13** and the amide analogues **21**, **22**, **26** (obtained from maleic and citraconic anhydrides) showed the most promise as acid-activatable prodrugs under mildly acidic conditions found in the cancer microenvironment. These prodrugs released 12−83% of emetine at pH 6.5 and 41−95% emetine at pH 5.5. Compounds **13** and **26** were further shown to exhibit increased cytotoxicity in PC3 cell culture medium that was already below pH 7.0 at the time of treatment.
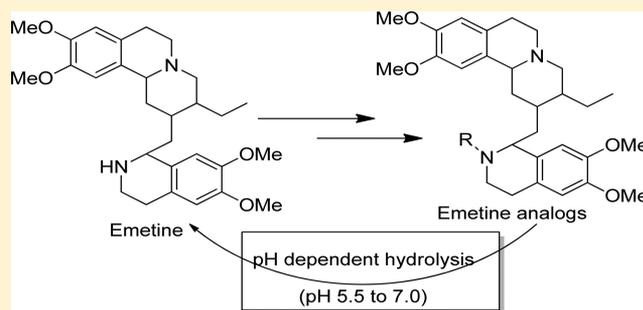
INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in men in the United States with approximately 240 000 cases annually.[1,2] About 33 000 American men die of prostate cancer each year with African American men having the highest death rate in the world.[2,3] Androgen ablation therapy has remained the gold standard first line treatment of metastatic prostate cancer since its discovery in the 1940s. While producing remarkable palliative benefit, androgen ablation is never curative and all men progress to a castration resistant state. Recently, several new agents have been approved for the treatment of castration resistant disease based on a few months of median survival improvement over placebo. While these results are encouraging, there remains an urgent need to develop new therapies for castration resistant prostate cancer.

One method for identifying new treatments is to look for older therapies that may have unique mechanisms of action that could be effective in cancer treatment. Emetine (**1**, Figure 1) is one such older agent that is a natural product alkaloid found in the root of *Psychotria ipecacuanha*.[4] It is the active ingredient in the ipecac root long used in traditional folk medicine as an emetic and expectorant.[5] Ipecac remains a staple in most households where it is used to induce vomiting in the event of accidental ingestion of toxic agents. Direct subcutaneous injection of emetine was, for a long time, also the treatment of choice for amoebiasis, amebic dysentery, and trypanosomiasis.[6]

While emetine has been shown to inhibit a number of important cell processes, its major mechanism of action appears to be related to its ability to inhibit ribosomal protein synthesis as first reported by Grollman[5] while Lietman later reported the same in the mitochondria.[7] As a protein synthesis inhibitor, its anticancer activities were investigated in several phase I−II clinical trials in a number of solid tumors between 1969 and 1974.[8−13] While clinical responses were observed in these studies, emetine was reported to have a very narrow therapeutic index, and dose dependent side effects such as muscle fatigue and cardiac toxicity were observed. These were the same side effects observed in the treatment of amoebiasis patients.[14,15]

On the basis of the toxicity observed in these clinical studies, development of emetine as an anticancer drug ceased.[16] Since then, this natural product has been used as an important tool in cell biology and pharmacology for in vitro studies that require inhibition of protein biosynthesis. These more recent studies have revealed the potency of emetine in modulating different biological pathways associated with cancer growth.[6] However, to further develop emetine-based therapeutics, an analogue must be developed that is only active upon reaching the tumor
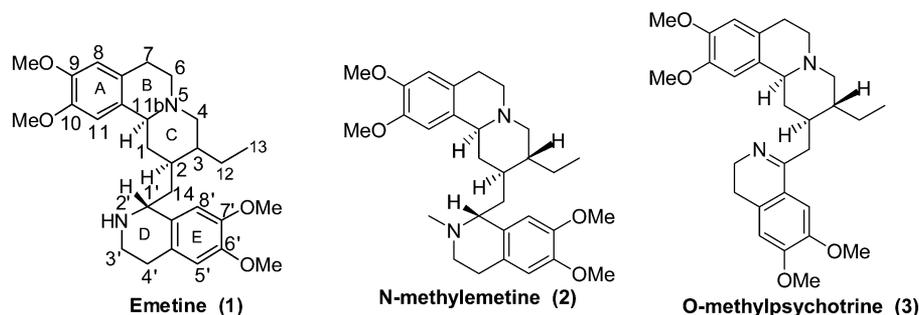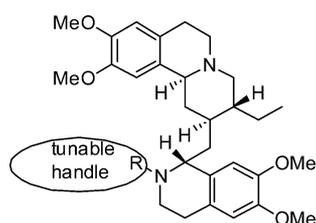
**Figure 1.** Chemical structure of emetine and some of its natural analogues.

microenvironment while relatively inactive in the general circulation and in normal tissues. Thus, to achieve this goal, it is important that we understand the effects of its structure and functional moieties on its biological activities (Figure 2).

Figure 2. General chemical structure of proposed emetine analogues with tunable handle.

An account of structure−activity relationship (SAR) studies on emetine and its biological activities done by various research groups has been recently reviewed.[6] The available SAR data demonstrate that a secondary amine in the N-2′ position of emetine is critical for protein synthesis inhibition. A relatively significant loss of protein synthesis inhibitory activity was observed in both *N*-methylemetine (**2**) and *O*-methylpsycho-trine (**3**); the hydrogen of the secondary amine might be involved in a crucial hydrogen bonding interaction of emetine to its targeted receptor.[17] In addition, a more recent conformational study reported the critical need for the region between the tricyclic system (A, B, C rings) and the bicyclic isoquinoline (D, E) system to be unoccupied for emetine and its analogues to be active.[18] Minimum energy conformational studies revealed that this region is occupied in inactive analogues while unoccupied in active analogues.[18]

These studies document that modification of the N-2′ position of emetine results in reduced biological activity including protein synthesis inhibition and toxicity. Replacement of the N-2′ secondary amine hydrogen with a bulkier group may also lead to conformational changes that will affect the region S (Figure 3) between the tricyclic and bicyclic systems. Consequently, an appropriate substituent in this position that could be selectively removed within the tumor microenviron-ment but remain stable in normal tissue could result in an emetine analogue with improved therapeutic index that could be useful as cancer therapy. In our efforts to develop emetine into clinically useful anticancer agent we have thus focused on chemical modification of the N-2′ position to (1) generate a functionally diverse library of emetine analogues with various hydrolyzable moieties (such as sulfonamides, urea, thiourea, carbamates, dithiocarbamate, and amide), which are also known for incorporating bioactivities into small molecules,[19−21] and (2) generate a set of prodrugs that will only be activated to
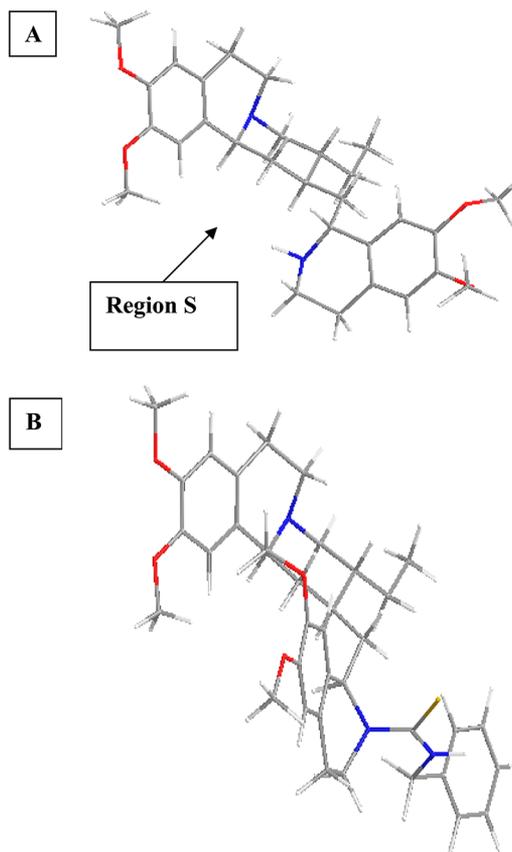
Figure 3. Energy minimized diagram of compounds **1** and **4**: (A) emetine (**1**) and (B) an N2′ derived thiourea analogue of emetine with benzyl substituent, compound **4**.

release emetine in the cancer cells or tumor microenvironment. The prodrugs could be activated either at the cancer microenvironment by exploiting the lower extracellular pH (pH ≈ 6.4−6.9) of some tumor cells compared to that of normal tissue[22−24] or the mild acidic conditions of the intracellular vesicles such as endosomes (pH ≈ 5.5−6) and lysosomes (pH ≈ 4.5−5).[25]

The present paper reports our initial studies on the synthesis and anticancer activities of novel bioactive emetine derivatives and acid-hydrolyzable prodrugs by chemical modification of the N-2′ position. The cytotoxic activities of these compounds were evaluated on androgen receptor positive LNCaP and negative PC3 prostate cancer cell lines.

■ RESULTS AND DISCUSSION

Evaluation of the Antiprostate Cancer Activity of Emetine. Because of lack of data on the cytotoxicity of emetine in prostate cancer cell lines, we began this study by first evaluating its anticancer potency in a representative androgen receptor positive LNCaP and androgen receptor negative metastatic PC3 and DU145 prostate cancer cell lines. Cell survival and proliferation were determined spectrophotometrically using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Supporting Information). For the biological studies, emetine was used as the dihydrochloride salt. Time and concentration dependent potency of emetine in this study revealed that emetine could be a novel antineoplastic agent in prostate cancer drug development (Table 1) if the therapeutic index is somehow improved. Emetine is equally potent in both androgen receptor positive and negative prostate cancer cell lines. As shown from the IC$_{50}$ values (concentration of drug that reduce the average growth value of the cancer cell lines by 50% relative to the average control growth value), cell death due to emetine relative to control was significant within the first 3 days of exposing the cells to emetine dihydrochloride hydrate with IC$_{50}$ values in the nanomolar range. Encouraged by this initial screening result with emetine in prostate cancer cell lines, we designed and synthesized thiourea, urea, sulfonamide, carbamate, dithiocarbamate, and amide derivatives of emetine via modification of the N-2′ position.

Table 1. Time and Concentration Dependent Cytotoxicity of Emetine in Prostate Cancer Cell Lines

	IC$_{50}$ (nM)		
exposure time	LNCaP	PC3	DU145
day 3	37.87 ± 0.76	35.13 ± 1.02	32.88 ± 0.99
day 5	27.82 ± 3.84	26.84 ± 2.27	25.27 ± 0.8
day 7	31.58 ± 2.42	29.43 ± 3.27	23.74 ± 1.22

Synthesis of Emetine Analogues 4−15 and 20−27. As depicted in Scheme 1a, efforts on the synthesis of thiourea analogues commenced with commercially available amines. Treatment of the appropriate amines with carbon disulfide in tetrahydrofuran in the presence of Et$_3$N resulted in an in situ generation of dithiocarbamic acid salt which on subsequent treatment with 4-toluenesulfonyl chloride as described by Wong and Dolman[26] gave the corresponding isothiocyantes in 74−93% yields. Reaction of the isothiocyanate with emetine dihydrochloride in CH$_2$Cl$_2$ in the presence of pyridine furnished the appropriate thiourea derivatives 4−6 in 47−51% yield.

The urea analogues 7−9 were synthesized in two steps (Scheme 1b). First, the requisite isocyanates were prepared by treating an appropriate amine with trichloromethyl chloroformate in CH$_2$Cl$_2$ in the presence of non-nucleophilic amine base 1,8-bis(dimethylamino)naphthalene.[27] A predetermined quantity of emetine dihydrochloride was then added to each isocyanate in CH$_2$Cl$_2$ in the presence of 4-dimethylaminopyridine (DMAP) to produce compounds 7−9 in 74−85% yield. Unlike the thiourea synthesis, the urea analogues were isolated in a relatively high yield.

The sulfonamide analogues 10−12 were obtained in 74−86% yield by treating emetine dihydrochloride with the appropriate sulfonyl chlorides in CH$_2$Cl$_2$ in the presence of 4-dimethylaminopyridine (DMAP) (Scheme 1c). This reaction progressed well with DMAP but not with triethylamine as base.
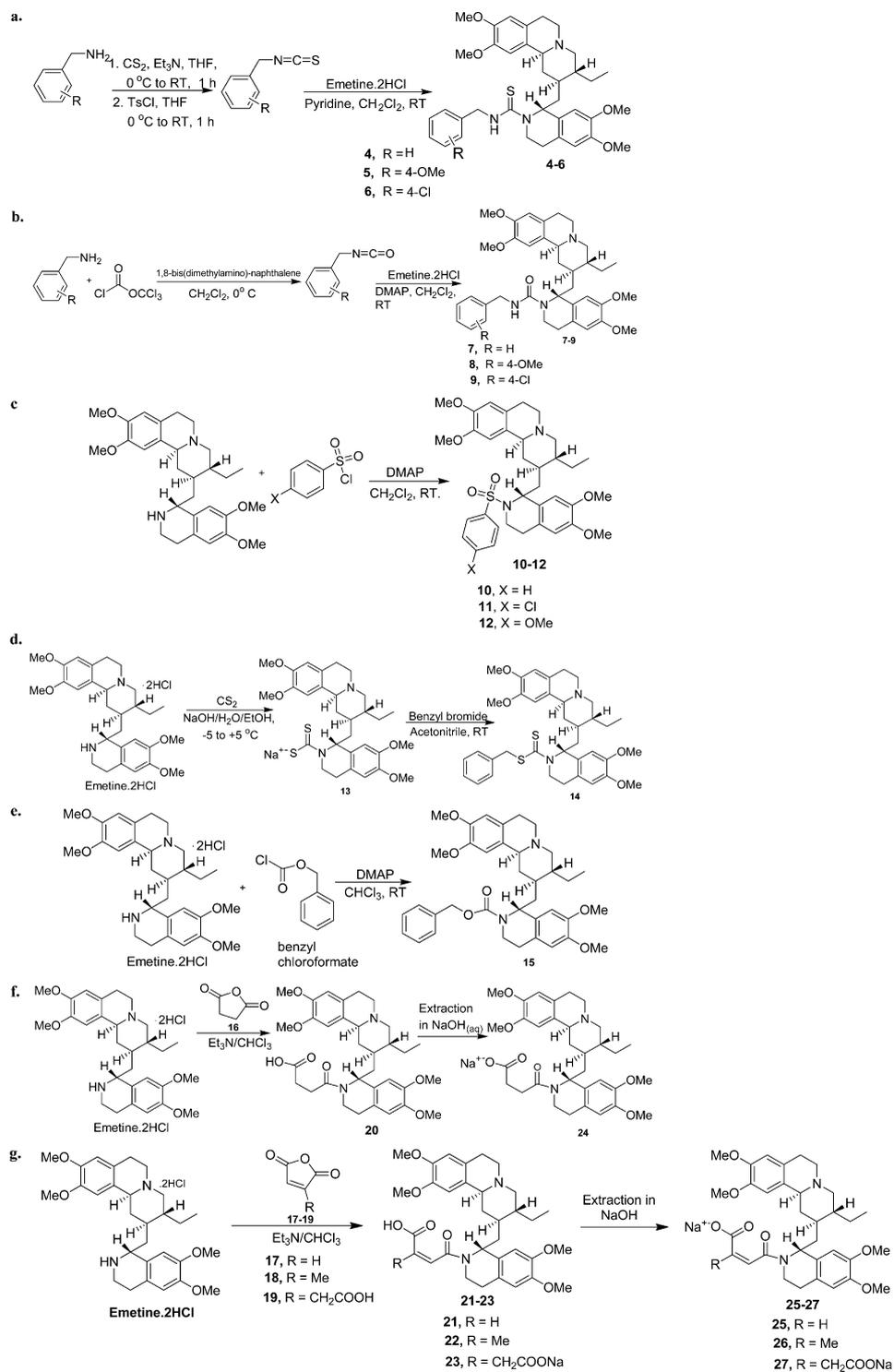
The preparation of the dithiocarbamate analogue 14 was achieved in two steps. Emetine dihydrochloride was reacted with carbon disulfide in ethanolic sodium hydroxide at −5 to +5 °C to produce the dithiocarbamic acid salt 13 which on treatment with benzyl bromide afforded 14 in 45% yield (Scheme 1d).

The carbamate analogue 15 was synthesized in 46% yield by reacting emetine dihydrochloride with benzyl chloroformate in the presence of at least 1 molar equivalent of DMAP in excess triethylamine using chloroform as solvent (Scheme 1e). Attempts to conduct this reaction in CH$_2$Cl$_2$, THF, and a number of other solvents were not very successful.

As stated earlier, we desire to target emetine directly to the cancer region in the form of prodrugs that will be activated only at the cancer microenvironment either intercellularly or intracellularly. To achieve this goal, we also synthesized pH-responsive amide analogues of emetine (20−27) from cyclic anhydrides (Scheme 1f and Scheme 1g). These analogues were synthesized by reacting emetine with appropriate anhydride in CHCl$_3$ in the presence of triethylamine to furnish the amide derivatives. Compounds 20−22 were isolated in 89−94% yield. To remove all the residual triethylamine from the cis-aconitylamide analogue (23), this analogue was treated with 0.1 N aqueous NaOH. Thus, the dicarboxylic acid 23 was converted to its disodium salt 27 in about 79% overall yield. Similarly, compounds 20−22 were converted to the respective sodium salts 24−26 with overall yield of 78−88% from emetine. With the amide analogues obtained as the sodium salts, 24−27, it was possible to do direct structure−activity relationship studies on the bioactivity and acid responsiveness of the four compounds.

Initial in Vitro Determination of the Antiprostate Cancer Potency of the Emetine Analogues. First, to determine the general effect of derivatizing emetine at the N-2′ on its cytotoxicity and to do structure−activity relationship studies on these synthetic analogues, we began by assaying their cytotoxicity in androgen receptor positive LNCaP and androgen receptor negative PC3 human prostate cancer cell lines. Six concentrations of each compound were tested on each of the two cell lines over a period of 7 days. We utilized the colorimetric MTT assay to determine the viable cells after the third, fifth, and seventh day of exposing the prostate cancer cell lines to the compounds. The anticancer potency of each compound on the seventh day measured as IC$_{50}$ value is reported in Table 2.

This study further confirms that derivatizing emetine at the N-2′ position to produce a tertiary nitrogen results in reduction of its cytotoxicity. The extent of reduction depends not only on the functional groups bonded to this position but also on the structural features of the substituents attached. Thus, the N-2′ position can serve as a tunable point where chemical modification can be carried out with the goal of generating emetine analogues and prodrugs that avoid the systemic toxicity associated with the parent compound. As observed for emetine, almost all the analogues have relatively potent cytotoxicity in both androgen receptor positive LNCaP and negative PC3 prostate cancer cells. The IC$_{50}$ values are below 10 μM except for those of compounds 9 and 10 in PC3 cell lines. Among the thiourea analogues (4−6), compound 6 with electron withdrawing chlorine atom at the para position is the most active (Table 2). However, a distinct preference for electron

Scheme 1. Synthesis of Emetine Analogues (4–15, 20–27)[a]

[a](a) Synthesis of thiourea derivatives of emetine (4–6) from isothiocyanates. The requisite isothiocyanates were prepared from corresponding amines. (b) Synthesis of urea derivatives of emetine (7–9) from isocyanates. The isocyanates were made from commercially available amines and trichloromethyl chloroformate. (c) Synthesis of sulfonamide derivatives (10–12) by reacting emetine with sulfonyl chlorides. (d) Synthesis of dithiocarbamate derivative of emetine (14) by the synthesis of dithiocarbamic acid salt (13) followed by S_N2 reaction between the salt and benzyl bromide. (e) Preparation of carbamate derivative (15) by reaction of emetine with benzyl chloroformate. (f, g) Preparation of amide derivatives (20–27) by treating emetine with appropriate cyclic anhydride.

withdrawing group over electron donating group is not too obvious, as the IC_{50} values for compound 5 with electron donating methoxy group are slightly lower than that of compound 4 in PC3. A consistent observation is that these

thiourea analogues have relatively lower IC_{50} values in LNCaP than in PC3 cell lines.

Similarly the urea analogues 7 and 9 exhibit more potency in LNCaP than PC3 as seen from the IC_{50} values; however, the

Table 2. In Vitro Cytotoxic Evaluation of Emetine Analogues 4−22 and 24−27 in LNCaP and PC3 Prostate Cancer Cell Lines Presented as IC$_{50}$ (in μM) after 7-Day Exposure

compd	LNCaP	PC3
4	2.057 ± 0.438	6.916 ± 0.071
5	3.388 ± 0.139	5.002 ± 0.307
6	1.590 ± 0.151	2.864 ± 0.066
7	2.115 ± 0.576	7.613 ± 0.681
8	7.839 ± 0.315	3.669 ± 0.796
9	5.411 ± 0.212	10.000 ± 0.512
10	2.263 ± 0.798	>10.0
11	2.692 ± 0.055	4.214 ± 0.435
12	3.153 ± 0.099	6.075 ± 0.105
13	0.079 ± 0.003	0.087 ± 0.005
14	1.970 ± 0.088	1.560 ± 0.290
15	3.241 ± 0.175	6.071 ± 0.144
20	2.409 ± 0.053	2.102 ± 0.172
21	6.781 ± 0.283	5.041 ± 0.087
22	0.368 ± 0.024	0.378 ± 0.010
24	2.451 ± 0.122	2.149 ± 0.131
25	8.901 ± 0.079	7.708 ± 0.151
26	0.458 ± 0.012	0.428 ± 0.008
27	5.465 ± 0.039	2.889 ± 0.123

urea analogue **8** with the para electron donating methoxy group exhibited distinctly better potency (IC$_{50}$, 3.669 μM) in the androgen receptor negative PC3 than androgen receptor positive LNCaP (IC$_{50}$, 7.839 μM). By comparison of the thiourea and urea analogues, it appears that without any substituent at the para position, replacement of the sulfur in thiourea by oxygen atom in the urea analogue does not significantly affect the cytotoxicity in either cell lines as shown by the similar IC$_{50}$ values obtained for thiourea derivative **4** (LNCap, 2.057 μM; PC3, 6.916 μM) and urea derivative **7** (LNCap, 2.115 μM; PC3, 7.613 μM).

All the sulfonamide analogues **10−12** also have higher potency (lower IC$_{50}$ values) in LNCaP than in PC3 but the range is very narrow in LNCaP, making it difficult to tell if the substituent at the para-position really affects the cytotoxicity. Compound **10** with no substituent on the sulfonamidophenyl ring is the least active in PC3 cell lines (IC$_{50}$ > 10).

Interestingly, the dithiocarbamic acid salt **13** is significantly more potent in both LNCaP and PC3 than the emetine analogues **4−12** discussed above. The seventh day IC$_{50}$ value is only about 3-fold higher than what we obtained for emetine. However, upon alkylation of **13** to the benzyl dithiocarbamate ester **14**, a significant reduction in potency measured by a higher IC$_{50}$ value of about 24-fold increase in LNCaP and 18-fold increase in PC3 (Table 2) was observed. The significance of this observation is further investigated in our prodrug development (Figures 4a,d and 6). We needed to know if **13** is acting by a different pharmacophore compared to emetine or if it was hydrolyzed in vitro and served as a prodrug for emetine. The carbamate ester **15** has a slightly lower potency (higher IC$_{50}$ value) relative to dithiocarbamate **14** in both cell lines (Table 2).

With the possibility that the better cytotoxicity of the dithiocarbamate salt **13** is due to its ease of hydrolysis, thus serving as an emetine prodrug, the maleic acid monoamide derivatives **21, 22,** and **25−27** were specifically designed as acid-activatable prodrugs of emetine while succinic acid monoamide derivatives **20** and **24** are not expected to be as

easily hydrolyzable. Maleic anhydride has been used for the reversible blocking or protection of amino groups of proteins and peptides in a reversible pH sensitive reaction.[28−30] Hydrolysis of the protecting maleic acid monoamide is known to occur under mildly acidic conditions by intra-molecular acid catalyzed hydrolysis which initially causes reversion to the amino group and maleic anhydride.[29−32] The maleic anhydride from the deprotection can then undergo further hydrolysis to maleic acid. A similar reversible blocking of amino groups of protein was reported for citraconic anhydride (a maleic anhydride derivative) and found to undergo faster hydrolysis than blocking with maleic anhy-dride.[33] A number of studies have employed maleic acid amide derivatives as acid-activatable linkers for daunomycin- and doxorubicin-macromolecule conjugates in targeted drug deliv-ery to tumor cells.[34−36] The *cis*-aconitic monoamide derivative of daunomycin has been reported as a pH activated prodrug that releases daunomycin under acidic pH but more stable at neutral pH.[34,37] Hence, the proposed maleic acid monoamide analogues (**21, 22, 25−27**) are expected to release emetine in the acidic cancer environment while remaining stable at the physiological pH range of 7.0−7.4.

Each amide with a carboxylic acid terminal (**20−22**) has a slightly higher cytotoxic activity (lower IC$_{50}$ value) than the corresponding sodium salt, **24−26** (Table 2). The citraconyl derivative **22** with a vinylic methyl substituent is the most active in the acid category of the amides (**20−22**), and its corresponding sodium salt **26** is the most active among the sodium salts (**24−27**). Though not as active as the dithiocarbamate salt **13**, compounds **22** and **26** are significantly more potent than emetine analogues **4−12** and **15**. The remaining amide analogues **20, 21, 24, 25,** and **27** show similar cytotoxicity levels as the other emetine analogues **4−12** and **15** in this study. As mentioned above, the citraconyl moiety in compounds **22** and **26** had been previously employed in reversible blocking of amino groups in proteins and gave satisfactory results in the ease of deblocking by hydrolysis of the amide bond in slightly acidic pH conditions. It is therefore possible that the citraconyl group in compounds **22** and **26** was hydrolyzed to emetine in the in vitro cancer microenvironment of the tissue culture medium. In light of this possibility, we further evaluated the time-dependent cytotoxicity of some of these emetine analogues in prostate cancer cell lines and studied their pH-dependent hydrolysis to emetine.

To obtain an understanding of the time-dependent cytotoxicty of some of the compounds and use the information to gain insight into a possible pH activated prodrug development for emetine, we studied the progress of the in vitro cytotoxicity of these emetine analogues over a 7-day period by comparing the IC$_{50}$ values obtained on the third, fifth, and seventh day after exposing the cells to the drugs. We wanted to know how long it took to reach the optimum cytotoxic activity for each drug compared to that for emetine and also to see if there is a progressive cytotoxicity common to each group of compounds over the 7-day period. For each compound, a similar progression in cytotoxicity is observed in both LNCaP and PC3. So we present the data obtained in PC3 cell lines in Figure 4. On the basis of these data, it is clear that the cytotoxicity of emetine reached an optimum by the third day of exposing the prostate cancer cells to emetine dihydrochloride. The fifth and seventh day studies gave results very close to that of day 3 (Figure 4a). An almost similar pattern was observed for the cytotoxic activity of thiourea
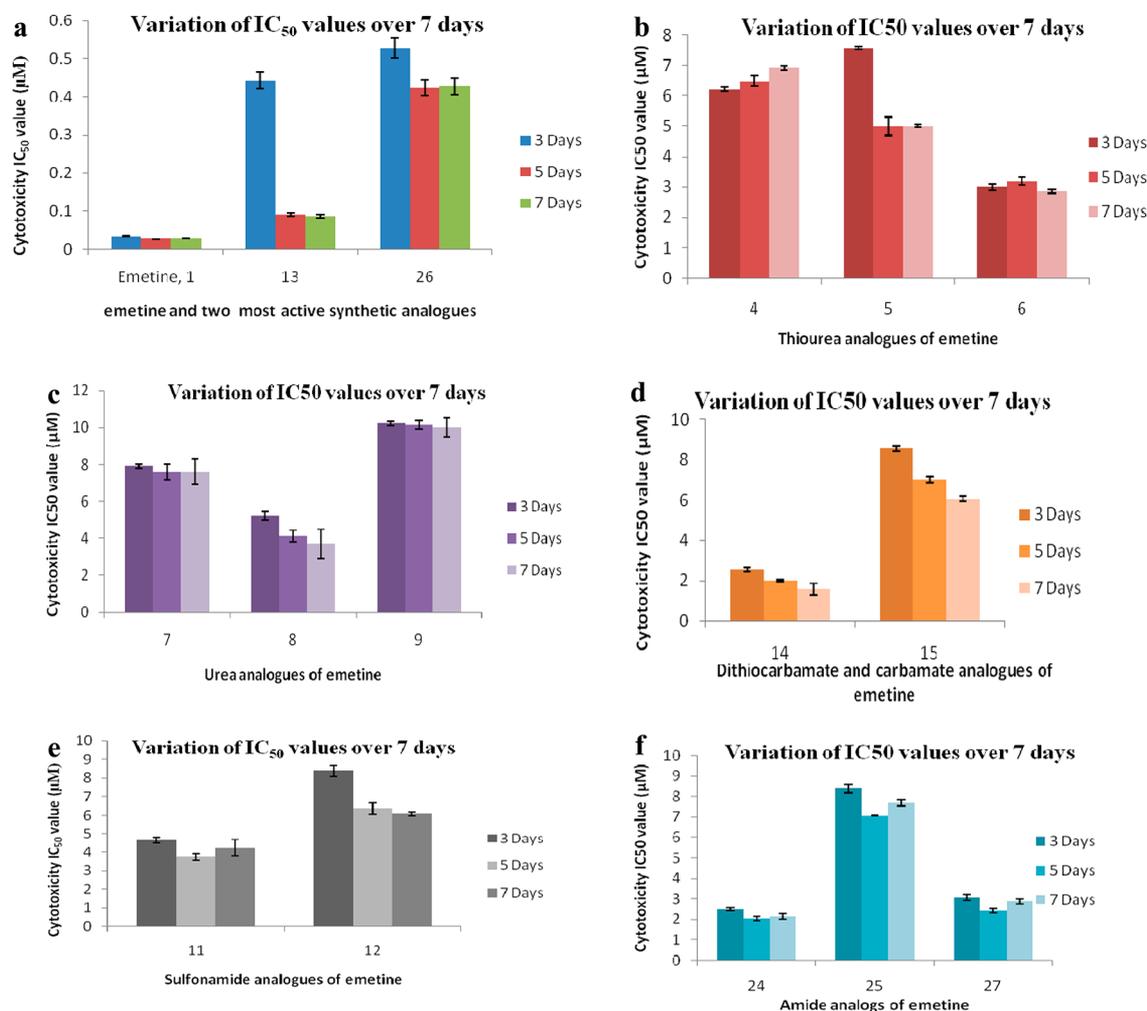
Figure 4. Variation of cytotoxicity (IC$_{50}$ values) of each compound over 7 days in PC3 cells. PC3 cell lines were incubated with six different concentrations of emetine dihydrochloride salt and its synthetic analogues for 7 days. MTT cell proliferation assay was carried out to determine the viable cells on each of the third, fifth, and seventh day of exposing the cells to each drug. The variation of cytotoxicity IC$_{50}$ values of emetine and its synthetic analogues with time over a 7-day exposure is shown in parts a−f.

analogue **6** and urea analogues **7** and **9** (Figure 4b,c). For these compounds, the IC$_{50}$ values also appear to reach an optimum by day 3 and the potency did not change much from day 3 to day 7. However, for compounds **5** (a thiourea, Figure 4b), **12** (a sulfonamide, Figure 4e), **13** (dithiocarbamate salt, Figure 4a), **26** (amide derivative, Figure 4a), and **24** (amide derivative, Figure 4f), an optimum IC$_{50}$ value was obtained by day 5 after exposure. The IC$_{50}$ value for each of these compounds on day 7 is similar to that of day 5. For urea analogue **8** (Figure 4c), dithiocarbamate ester **14** (Figure 4d), and carbamate ester **15** (Figure 4d), there appear to be a progressive decrease in IC$_{50}$ values (increase in cytotoxicity) from day 3 to day 7. The remaining analogues, **4**, **11**, **25**, and **27**, appear to show very close IC$_{50}$ values for days 3, 5, and 7.

No consistent pattern is observed in each class of compounds, and we believe that in addition to functional moieties, the conformation of each analogue most likely affected its potency. A study on the effect of conformation of these analogues, its implication on the size of space "S" between the tricyclic ABC rings and bicyclic DE rings (Figure 3), and correlation of these to anticancer activities in emetine analogues is currently being planned. Of much interest in the study presented here, however, is the big increase in cytotoxicity

observed for compound **13** between day 3 and day 5. In line with our design of an emetine prodrug, we were interested in understanding if this compound was acting as a prodrug and thus not as potent by day 3. And if so, was compound **13** hydrolyzed to emetine by day 5 such that the potency observed after day 5 resulted from emetine? To have an insight into this possibility, we used HPLC to study the acid-catalyzed release of emetine from this compound and some of the other potentially pH-activatable analogues (**21**, **22**, **25**, **26**, and **27**). It is important to mention at this point that all the synthetic emetine analogues presented in Scheme 1 are hydrolyzable with different degrees of responsiveness to pH change and the time required. However, compounds **13**, **21**, **22**, **25**, **26**, and **27** are expected to be more easily hydrolyzable under mildly acidic environment as discussed above. The results of the evaluation of the pH-responsiveness of **13**, **21**, **22**, **25**, **26**, and **27** by HPLC are presented in the following section and in Figure 5.

Evaluation of pH-Responsiveness of Compounds 13, 21, 22, and 25−27 by HPLC. Relative rates of hydrolysis of these compounds over a 48 h period were evaluated using the percent emetine released from the acid catalyzed hydrolysis of **13**, **21**, **22**, and **25−27**. The compounds were incubated in aqueous phosphate buffer at pH 5.5, 6.5, and 7.4 at 37 °C over
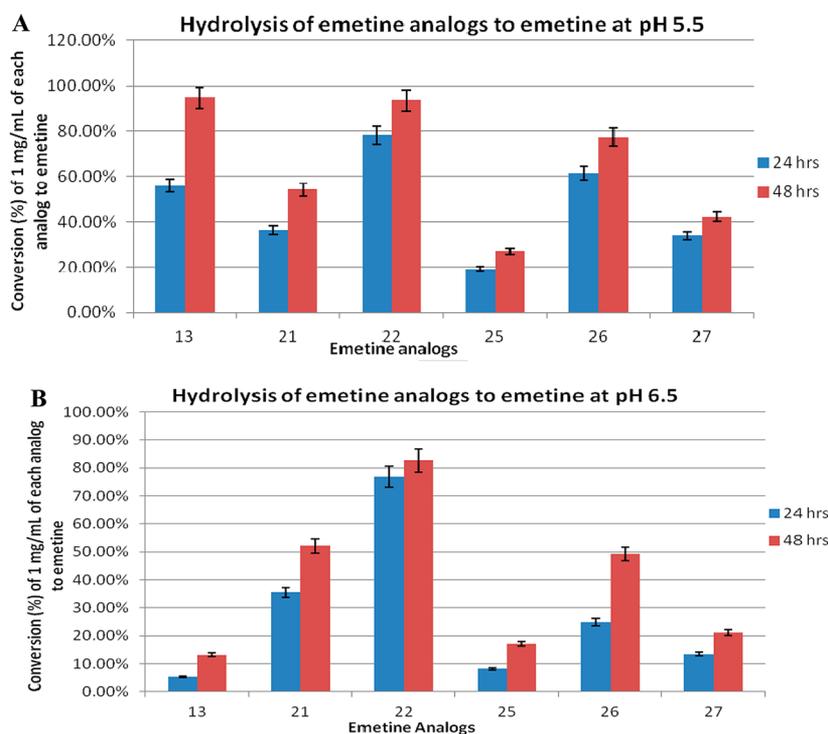
Figure 5. Quantitative analysis of emetine released in the pH responsive hydrolysis of emetine analogues. (A) Hydrolysis of emetine analogues to emetine at pH 5.5. Percent conversion indicates percent emetine in the sample mixture after pure analogues are incubated at pH 5.5 phosphate buffer at 37 °C for 24−48 h. (B) Amount of emetine released at pH 6.5, with the same study as in (A) carried out at pH 6.5.

a 48 h period. High performance liquid chromatography (HPLC) was employed to analyze the samples and quantify how much emetine was released. The data are summarized in Figure 5. The compounds whose hydrolysis to emetine is shown in Figure 5 are all 100% stable at physiological pH 7.4 up to a 5-day exposure. All the compounds studied here (**13**, **21**, **22**, **25**, **26**, and **27**) were activated to emetine at pH 5.5 over a 48 h period, although to different extents. Percent hydrolysis to emetine was found to drop at the less acidic pH 6.5. These results are consistent with earlier reports of the hydrolysis of the monoamides of maleic anhydride derivatives outlined above.[29−34,37] Hence, amide analogues synthesized from maleic anhydride and its derivatives in this study showed great promise as seen particularly in **21**, **22**, and **26** which release about 50%, 80%, and 50% emetine, respectively, within 48 h at pH 6.5. In addition, the free acid analogues **21** and **22** appear to be more pH-responsive and sensitive than the corresponding sodium carboxylate salts **25** and **26**. The pH responsiveness of the sodium dithiocarbamate salt **13** is higher at pH 5.5 than at 6.5, producing about 94.5% hydrolysis within 48 h at pH 5.5 but only 13.2% over the same period at pH 6.5. The hydrolysis of **13** appears to be slower at pH 6.5, and it is therefore conceivable that there is an increase in the amount of emetine released between day 3 and day 5 in the in vitro study, thus making this compound show optimum activity by day 5. A brief evaluation of the other emetine analogues **4−12**, **14**, and **15** shows that they are stable at these slightly acidic pH over the time period studied. Taken together, these data demonstrate that pH-responsiveness of **13**, **22**, and **26**, resulting in release of emetine, may be playing a significant role in their increased cytotoxicity (lower IC_{50} values) observed after day 3 when the pH of growth medium has probably dropped to between 6.0 and 7.0 (Figures 4a, 5, and Table 1). These results encouraged further in vitro studies of the activation of two of these

potential prodrugs (**13** and **26**) in cell culture medium under slightly acidic pH (6.0−6.9).

In Vitro Cytotoxic Studies of Compounds 13 and 26 in PC3 Cell Line under Pre-Established Acidic Cancer Cell Culture Medium (pH < 7.0). Upon establishing the release of emetine from these compounds under mildly acidic condition, we rationalized that we could increase the potency of the pH-responsive analogues if we could adapt the cancer cell to an acidic medium so that the pH of the cell culture is already below 7 at day 0. To investigate this, we selected compounds **13** and **26**, which are two of the three most cytotoxic pH-responsive analogues.

To accomplish our objective here, we first needed to establish the range of pH changes of the PC3 cell lines in our in vitro studies over a 7-day period. Hence, we investigated the effects of the metabolism of PC3 cell lines on change in pH of growth medium without any drugs, and the result is presented in Figure 6. This study showed that the metabolic activities of the PC3 cancer cells caused a fall in the pH of the growth medium from 7.4 on day 0 to 7.05 on day 3 and 6.66 by day 7 (Figure 6).

In our initial in vitro cytotoxic studies on these compounds, a gradual reduction in the pH of the growth medium was observed as the cancer cells metabolized over a 7-day period (Figure 6). We therefore reasoned that it is possible to adapt the prostate cancer cells to low pH between 6.5 and 7.0. Hence, confluent PC3 cell lines were left incubated at 37 °C until the growth medium attained a low pH of 6.7−7.0. The cells were further passaged twice into RPMI-1640 medium (pH 6.7−7.0) to allow them to adapt to this low pH environment. These cancer cells were then suspended in growth medium buffered at pH 6.8 using KH_2PO_4 and plated in a 96-well plate. Cells from the same passage were also plated in normal RPMI-1640 growth medium of pH 7.4. The growth of PC3 cell lines under
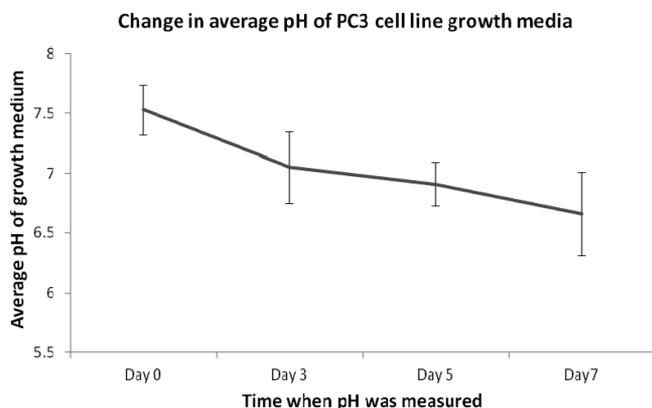
Figure 6. Reduction of average pH of growth medium over 7 days due to the metabolism of PC3 prostate cancer cell lines. Cell suspension was made at a density of 2000 cells per 100 μL and then plated in a 96-well plate at a density of 2000 cells/well. Medium was drawn off the wells on day 0, day 3, day 5, and day 7 of incubation. pH was measured and average pH calculated.

these two pH conditions was monitored over a 5-day incubation period. They both gave a comparable growth curve (Figure 7). Thus, this became our in vitro model for performing a pH-responsive prodrug activation assay.
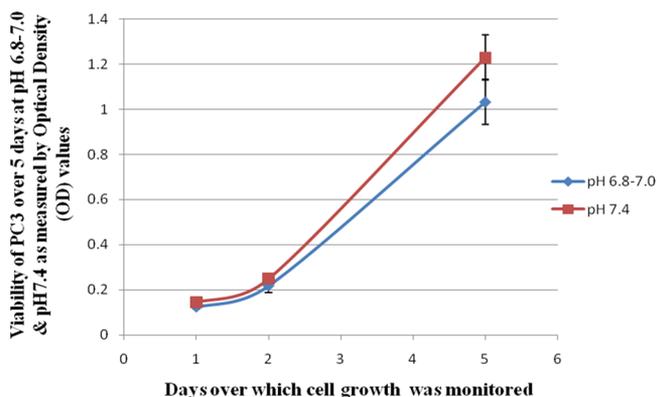
Figure 7. Comparison of growth of PC3 cell lines in growth medium of pH 6.8−7.0 and pH 7.4. These cells were adapted to growth in low pH medium and then plated in growth medium of pH 6.8−7.0 and pH 7.4 for comparison. MTT cell proliferation assay was done on each of day 1, day 2, and day 5. Growth of cells under these two conditions is comparable.

Prostate cancer cell lines already adapted to growth under this slightly acidic pH were thus plated at a higher density of 15 000 to 20 000 cells/well in growth medium of pH 6.8. Cells were incubated for 48 h to give room for metabolism that might lead to further lowering of pH before drug treatment. Cells were then treated with six different concentrations of each drug (**13** or **26**) prepared in a growth medium of pH 6.8. Emetine dihydrochloride was used as a positive control. Because of cancer cell metabolism, the average pH gradually decreased further to as low as 6.4 by the fifth day of this study. As expected, the difference in the cytotoxicity of **13** and **26** compared to that of emetine reduced drastically (only about 2-fold difference from emetine) at pH < 7.0. On the other hand, there is more than 16-fold difference in the cytotoxicity of **13** and **26** relative to emetine at pH 7.4 (Table 3). The increased cytotoxicity of these prodrugs (**13** and **26**) at pH less than 7.0

**Table 3. In Vitro Activation of pH-Responsive Representative Emetine Prodrugs 13 and 26**[a]

compd	IC$_{50}$ fold at pH 7.4[b]	IC$_{50}$ fold at pH < 7.0[b]
13	16.5	1.9
26	16.8	2.5

[a]Measured by change in ratio of cytotoxic IC$_{50}$ of each drug compared to that of emetine on day 5 in PC3 cell lines under different pH conditions. [b]IC$_{50}$ fold at each pH is determined as (IC$_{50}$ of each drug)/(IC$_{50}$ of emetine).

is thus in agreement with their activation to emetine under slightly acidic environment. Hence, it appears that emetine is the major cytotoxic agent when these compounds are subject to pH < 7.0. In addition, the results at pH 7.4 compared to pH < 7.0 indicate that these compounds are far more activatable in the acidic cancer environment, thus establishing these compounds as potential prodrugs of emetine. In order to carry out an initial assessment of the relative toxicity of the prodrugs **13** and **26** compared to emetine, we opted for in vivo toxicity studies in healthy mice.

In Vivo Toxicity Study To Establish the Safety of Emetine, 13, and 26 in Mice. The dose dependent side effects associated with the use of emetine usually culminates in lethality. To establish the safety of the N-2′ derived analogues developed in this study, we further evaluated the in vivo toxicity of emetine and compounds **13** and **26** in healthy mice.

These compounds were administered to the mice intravenously (iv). A single dose of emetine at 100 mg/kg body weight killed all three mice within 48 h. However, no sign of toxicity was shown in the mice that received 100 mg/kg body weight of compounds **13** and **26** daily for 5 days. To further investigate the safety of these compounds at 100 mg/kg, we determined the change in the body weight of these mice and that of the mice in the control group (mice that received just 1% DMSO in saline) within 48 h (Figure 8). The percent
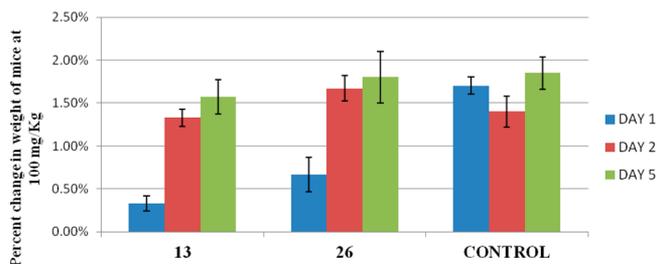
Figure 8. Percent weight change in mice that received 100 mg/kg body weight of compounds **13** and **26** and the control group over a 5-day daily dosing.

weight change observed within 48 h in the mice that received analogues **13** and **26** was very similar to that obtained for the control group (Figure 8). This indicates that this small weight change is not due to the toxicity effect of these drugs. The mice were further studied for 3 weeks, and no noticeable effect of toxicity was observed in them. To further establish the safety of one of the top pH-responsive prodrugs of emetine compared to emetine, we needed a dose of emetine that is not immediately lethal to the mice (i.e., does not result in death of the mice). To this end, we found that a lower dose of 33 mg/kg body weight of emetine did not kill the mice within the period of study. Hence, each mice group was given 33 mg/kg of emetine and prodrug **13**. Though mice that received 33 mg/kg of emetine

did not die within 48 h, these mice appeared slightly lethargic and weight loss was observed (Figure 9). A weight loss of 4.5%,
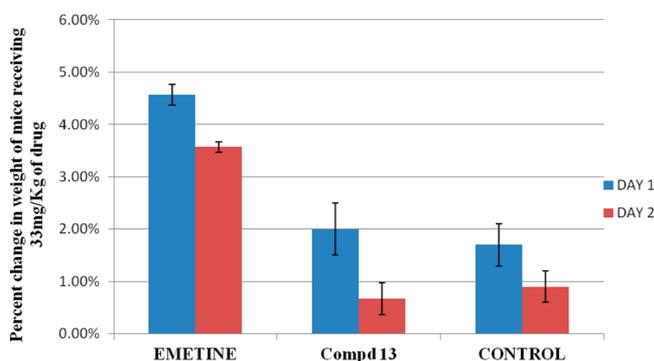
Figure 9. Percent change in weight of mice that received emetine and compound **13** at a dose of 33 mg/kg body weight.

2.0%, and 1.7% were observed in mice that received emetine, prodrug **13**, and control (1% DMSO in saline), respectively, within 24 h. Over a 48 h period, mice that received emetine at 33 mg/kg showed an average weight loss of about 3.6% and appeared lethargic while those that received 33 mg/kg of **13** showed a weight loss of 0.67% but appeared healthy with no noticeable signs of toxicity (Figure 9). Within 48 h of this daily dosing, the percent weight loss in mice that received emetine was about 4 times the weight loss in control mice, while there was no significant difference in weight loss between mice injected with prodrug **13** and the control group (Figure 9).

These results indicate that the toxicity associated with the use of emetine has been significantly reduced if not removed in these pH-responsive prodrugs. The drugs are therefore considered safe for further in vivo efficacy studies.

In summary, these results suggest that an appropriately designed emetine analogue could become a valuable cancer chemotherapy. The vital role of the N-2′ secondary amine is seen in the reduced cytotoxicity of all the N-2′ derived analogues. These analogues can be hydrolyzed to emetine at variable rates that depend on the substituent at the "tunable handle". It is also vital to note that these compounds are relatively stable at pH 7.4, indicating that emetine will most likely not be released in the blood or the environment of normal tissue as demonstrated in the nontoxic nature of two of these prodrugs relative to emetine in healthy mice.

CONCLUSION

Overall, in an attempt to improve the therapeutic index of the natural product emetine, we have developed several N-2′ derived analogues of emetine and they were assayed in both androgen receptor positive and negative prostate cancer cell lines. The data obtained clearly confirmed that the secondary amine of the N-2′ position of emetine plays a crucial role in its cytotoxic activity. All chemical modifications of this position to thiourea, urea, dithiocarbamate, carbamate, sulfonamides, and amides resulted in various analogues that are almost 5−400 times less potent than emetine. This is obviously a starting point for designing an emetine prodrug that will avert the systemic toxicity associated with its therapeutic use and thus improve its therapeutic index. The current study utilized synthetic analogues of emetine that are sensitive to pH change for their controlled activation. The water-soluble sodium dithiocarbamate salt **13** and the amide analogues **21, 22, 26**

(obtained from maleic and citraconic anhydrides) showed the most promise as potential pH-sensitive prodrugs for activation by the slightly acidic cancer microenvironment with significant stability at physiological pH. The results obtained from the compounds investigated showed that the N-2′ position is "tunable" for controlled release of emetine. The in vitro model of slightly acidic prostate cancer microenvironment was also developed and employed in in vitro activation of potential prodrug in this study. Further, the relative safety, in health mice, was demonstrated in vivo for two of the prodrugs **13** and **26** compared to emetine. Our findings in this preliminary study are a significant foundation for further in vivo efficacy and pharmacokinetic studies, as well as further efforts toward the design of new synthetic analogues of emetine in prodrug development.

ASSOCIATED CONTENT

Ⓢ Supporting Information
(1) Details of the analytical instrument used, (2) experimental details of all the intermediates and analogues of emetine, (3) experimental details of the biological assays, (4) further details on the pH responsiveness of emetine analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RPMI, Roswell Park Memorial Institute; LNCaP, androgen-sensitive human prostate adenocarcinoma; PC3, androgen-insensitive human prostate cancer cell lines; DU145, androgen-insensitive human prostate cancer cell lines

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