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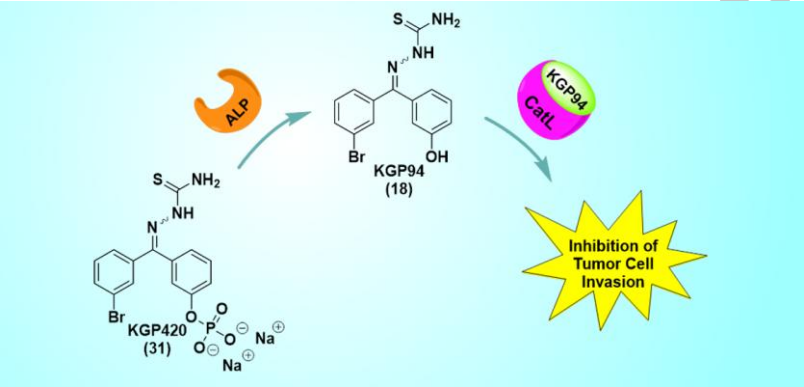

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ABSTRACT

The magnitude of expression of cathepsin L, often upregulated in the tumor microenvironment, correlates with the invasive and metastatic nature of certain tumors. Inhibition of cathepsin L represents an emerging strategy for the treatment of metastatic cancer. A potent, small-molecule inhibitor (referred to as KGP94) of cathepsin L, and new KGP94 analogues were synthesized. (3,5-Dibromophenyl)-(3-hydroxyphenyl) ketone thiosemicarbazone (**22**), with an IC_{50} value of 202 nM, exhibited similar inhibitory activity against cathepsin L compared to KGP94 (IC_{50} = 189 nM). Due to limited aqueous solubility of KGP94, a water-soluble phosphate salt (KGP420) was prepared in order to facilitate future in vivo studies. Enzymatic hydrolysis with alkaline phosphatase (ALP) demonstrated that the phosphate prodrug, KGP420, was readily converted to the parent compound, KGP94.

The discovery and development of effective therapeutic regimens that target cancer metastasis remains an urgent and largely unmet need. Metastasis is a significant contributing factor in over ninety percent of deaths attributed to cancer.[1] The sequence of steps involved in the metastatic process associated with tumor cells includes invasion of these cells from the primary tumor into the surrounding tissue, intravasation into the circulatory system, extravasation from the circulatory system, and establishment of a secondary tumor.[1–3] One promising strategy towards the development of anti-metastatic agents involves targeting one or more members of the papain family of cysteine protease cathepsins (comprised of 11 members: B, C, F, H, K, L, O, S, V, W, and X/Z) with small-molecule inhibitors.[4–8] Cathepsins aid in the invasion and migration of tumor cells through the degradation of proteins comprising the extracellular matrix including collagen,[9–15] fibronectin,[9,10,16,17] and laminin.[9,10,17] Elevated levels of cathepsins L, B, H, X, and S have been detected in several cancer types including breast, prostate, brain, colorectal, and lung cancers.[18,19] Moreover, increased expression of these cathepsins correlates to poor prognosis in breast, ovarian, colorectal, brain, lung, and head and neck cancers.[18,19] Decreased tumor volume and increased survival rates were observed in a mouse model upon treatment with the pan-cathepsin inhibitor JPM-OEt in combination with cyclophosphamide, an established chemotherapeutic agent.[20]

In addition to the role that these enzymes play in metastasis and migration of cancer cells through degradation of components of the extracellular matrix, cysteine cathepsin proteases have been implicated as targets for osteoporosis,[21] rheumatoid arthritis,[22] atherosclerosis,[23] and diseases of the immune system.[24] Cathepsin inhibitors as drug candidates that have advanced in the pharmaceutical pipeline for the treatment of various diseases include VBY-825 (Virobay),[25,26] Odanacatib (Merck),[27–29] LY3000328 (Eli Lilly),[30,31] (Figure 1).

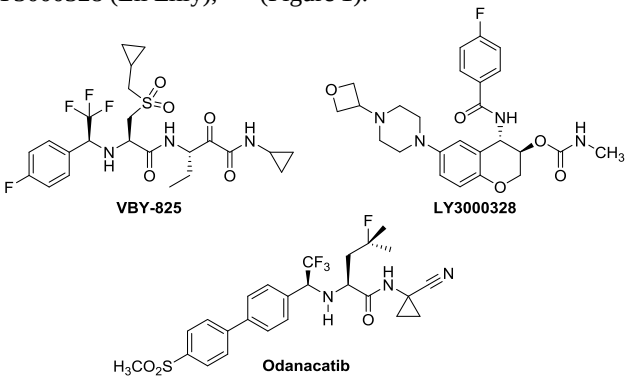

Figure 1. Sampling of cathepsin inhibitors recently described in the pharmaceutical pipeline

Abbreviations: CatL, cathepsin L; HUVECs, human umbilical vein endothelial cells; SAR, structure-activity relationship; MMP, matrix metalloprotease; ALP, alkaline phosphatase.

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VBY-825 (Virobay), a pan cysteine protease inhibitor targeting the treatment of liver fibrosis, incorporates a carbonyl warhead into a peptide-like backbone allowing for reversible inhibition of targeted cathepsins.[25,26] Odanacatib (Merck), a nitrile based inhibitor, targets cathepsin K for suppression of bone resorption in osteoporosis.[27–29] LY3000328 (Eli Lilly), a noncovalent cathepsin S inhibitor, targets the treatment of abdominal aortic aneurysm. [30,31] Although significant progress has been achieved towards the development of cathepsin inhibitors as therapeutic treatment options, no FDA approved drugs currently exist in this area. Additionally, small-molecule agents that specifically target the inhibition of cathepsin L for the treatment of pathological processes currently in clinical trials remains an unmapped frontier open to exploration.

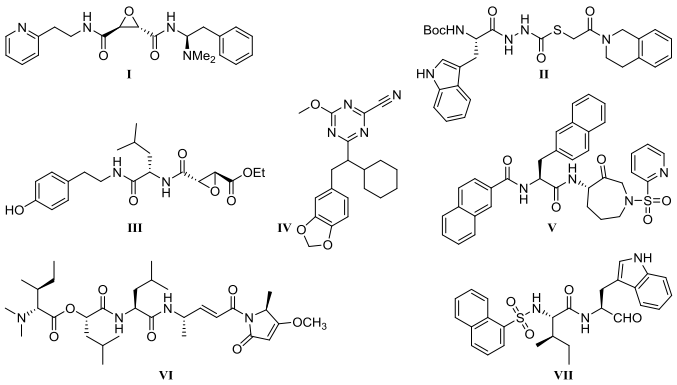


Figure 2. Representative potent covalent inhibitors of CatL.

Small-molecule inhibitors of cathepsin L have been synthesized that incorporate a variety of electrophilic moieties (warheads) capable of interacting with the catalytic site residue Cys25 (Figure 2). Warheads which undergo covalent bonding with the Cys25 thiolate of cathepsin L include the epoxide in Clik 148 (**I**),[32] the carbonyl of thiocarbazate **II**,[33] the epoxide in JPM-OEt **III**,[34,35] the nitrile in the triazine analogue **IV**,[36] the cyclic carbonyl in azepanone **V**,[37] the α,β-unsaturated amide of gallinamide A **(VI)**,[38,39] and the aldehyde of the *N*-(1-naphthalenylsulfonyl) peptide derivative **VII**[40] (Figure 2). Cruzain, a cathepsin L-like cysteine protease found in *Trypanosoma cruzi*, is targeted for the treatment of Chagas' disease. Initial studies towards the development of cruzain[41,42] inhibitors led to the discovery of cathepsin L inhibitors bearing the thiosemicarbazone moiety which contain an electrophilic thiocarbonyl warhead. Building on these results, we embarked on a structure-activity relationship (SAR) guided program designed to incorporate the thiosemicarbazone moiety within appropriately functionalized benzophenone, pyridine, thiophene, fluorene, thiochromanone, benzothiepine, and dihydroquinoline molecular scaffolds. A focused small library of cathepsin inhibitors resulted from these studies,[43–51] from which a sub-set of 42 molecules demonstrated IC_{50} values below 500 nM (Figure 3).

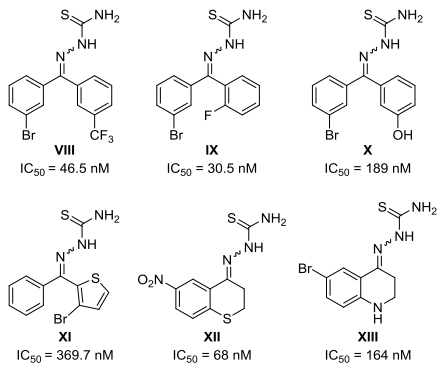
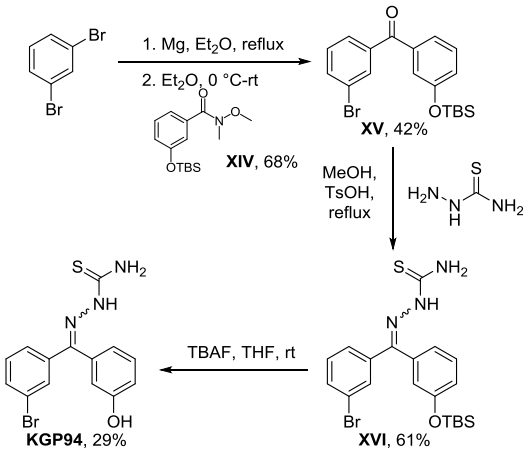


Figure 3. Representative examples of previously described thiosemicarbazone based inhibitors of CatL.

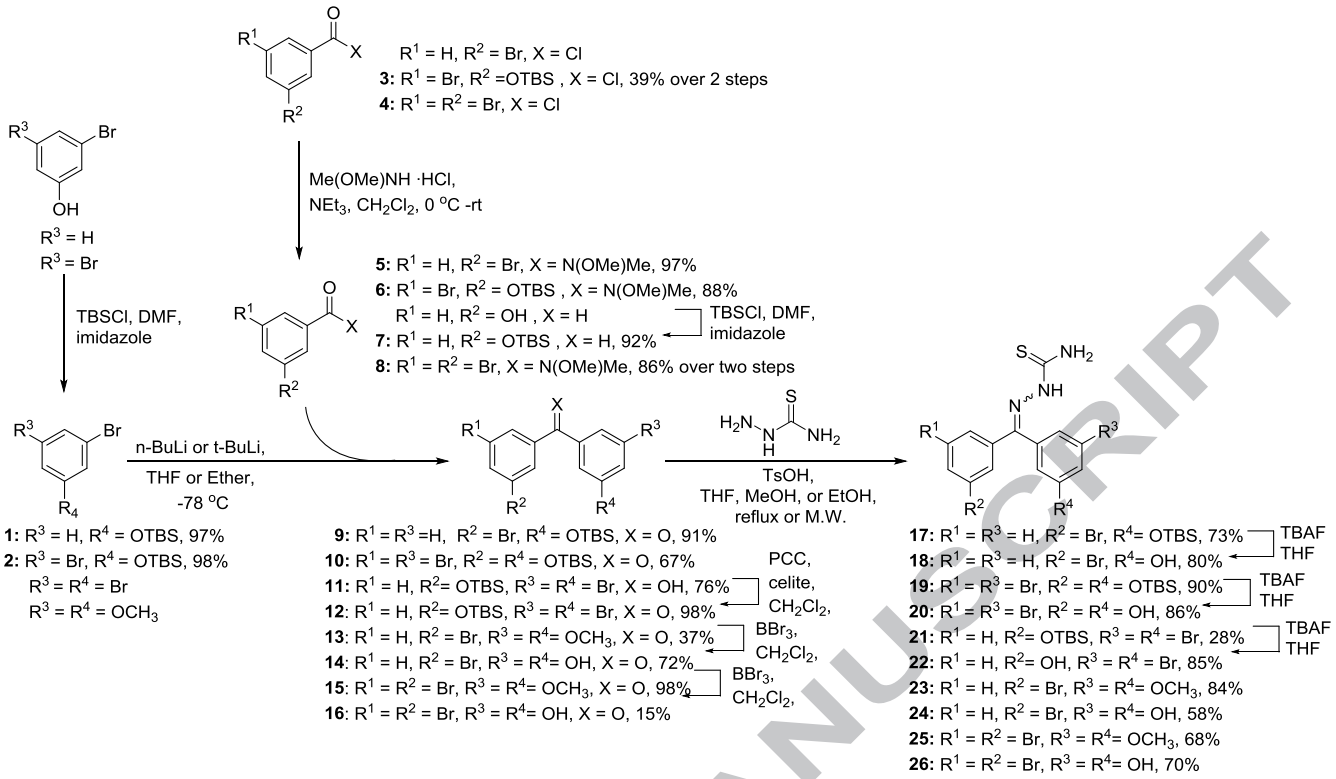
A lead compound (referred to as KGP94), which is a slowly reversible, time-dependent inhibitor of cathepsin L, emerged from this small library of structurally diverse thiosemicarbazone analogues.[43,46,47] Low cytotoxicity against human umbilical vein endothelial cells (HUVECs), the ability to inhibit the invasive and migratory potential of both PC-3ML (prostate cancer cell line) and MDA-MB-231 (breast cancer cell line) in vitro, and the ability to reduce metastatic tumor burden and increase survival rate in PC-3ML tumor bearing mice has led to the identification of KGP94 as a pre-clinical candidate for potential development as an anti-metastatic agent, functioning through a potent inhibition of cathepsin L.[43,47,52] Since KGP94 has limited solubility in water, it proved desirable to prepare a water-soluble prodrug salt to further the pre-clinical development of this promising agent. Fortunately, KGP94 bears a phenolic hydroxyl group which provides a convenient molecular handle for the introduction of a bioreversible ester linkage in order to improve aqueous solubility. Recently, we have reported the synthesis of the phosphate prodrug OXi8007, a vascular disrupting agent currently in pre-clinical studies for the treatment of cancer.[53] Installation of a phosphate prodrug salt for the purpose of increasing aqueous solubility of FDA approved drugs intended for oral or parental administration has been successfully demonstrated by fosfpropofol, fosamprenavir, and fosfluconazole.[54]

In addition to the synthesis of a water-soluble phosphate prodrug of KGP94, several analogues of this parent, lead compound were also prepared. Previous SAR studies related to thiosemicarbazone inhibitors based on the benzophenone molecular scaffold highlighted the importance of the 3-bromophenyl moiety.[45,46] The extended series of benzophenone-based thiosemicarbazone inhibitors incorporated both *m*-hydroxy and *m*-bromo substituents. Our initial synthetic route[46] to KGP94 (Scheme 1) utilized the addition of 3-bromophenylmagnesium bromide to the corresponding Weinreb amide **XIV** to afford (3-bromophenyl)(3-hydroxyphenyl)methanone (**XV**), which was condensed with thiosemicarbazide followed by deprotection of silyl ether **XVI**. However, HPLC analysis of the final product revealed the presence of a trace amount of an impurity in which the bromine atom had been replaced by a hydrogen atom.[46] Replacement of bromine by hydrogen likely occurred during the halogen-metal exchange reaction since excess magnesium was used to prepare benzophenone (3-bromophenyl)-(3-hydroxyphenyl)-methanone (**XV**).



Scheme 1. Previously reported synthetic route towards KGP94[46]

In an effort to avoid impurities in which the Br atom was replaced by a hydrogen atom, KGP94 and analogues were synthesized through a revised route (Scheme 2). Instead of using 1,3-dibromobenzene as the precursor to the organometallic

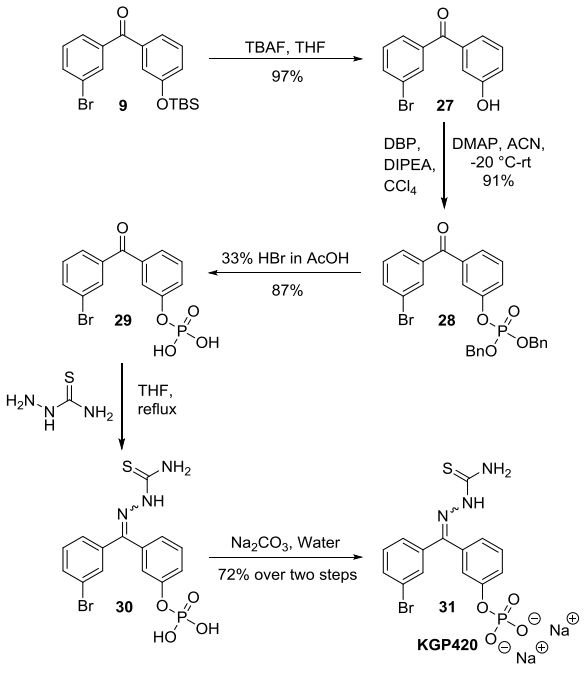


Scheme 2. Improved synthetic route towards KGP94 and analogues 20, 22, 23-26

reagent for the synthesis of KGP94 (**18**), a protected *m*-bromophenol **1** was reacted with *n*-butyllithium to form the intermediate organolithium reagent, which was reacted with Weinreb amide **5** to afford the desired functionalized benzophenone **9**. Benzophenone intermediates **10** and **12** were synthesized in a similar manner by reacting the appropriately substituted aromatic ring with *n*-butyllithium followed by the addition of Weinreb amide **6** to afford ketone **10** or the addition of aldehyde **7** followed by oxidation with PCC to afford ketone **12**. Condensation of benzophenone intermediates **9**, **10**, and **12** (separately) with thiosemicarbazide followed by desilylation with TBAF afforded target thiosemicarbazone analogues KGP94 (**18**), **20**, and **22**. HPLC analysis indicated no trace amount of the impurity in which bromine was replaced by hydrogen in the final product for KGP94 (**18**).

Synthesis of dimethylresorcinol and resorcinol analogues **23-26** utilized commercially available 1-bromo-3,5-dimethoxy benzene as a starting material to form an intermediate organolithium reagent which upon reaction with Weinreb **5** or **8** afforded ketones **13** and **15**, respectively (Scheme 2). Demethylation of these 3,5-dimethoxybenzophenone intermediates **13** and **15** with boron tribromide afforded 3,5-dihydroxybenzophenones **14** and **16**. Subsequent condensation of ketones **13-16** with thiosemicarbazide (separately) under microwave irradiation generated target thiosemicarbazone analogues **23-26**.

In order to increase the aqueous solubility and possibly the bioavailability of KGP94 (**18**), a water-soluble phosphate prodrug salt was prepared through phosphorylation of the phenolic moiety (Scheme 3). Initial attempts to incorporate the dual thiosemicarbazone and phosphate moieties were met with difficulty and included phosphorylation of (3-hydroxyphenyl)(phenyl)ketone thiosemicarbazone (used as a model system) which led to multiple products including benzylated and debenzylated side products. Attempts to deprotect dibenzyl(3-(3-bromobenzoyl)phenyl) phosphate using catalytic hydrogenation led to multiple products and with longer reaction times, in addition to removal of the benzyl groups, the carbonyl group was reduced to its corresponding methylene.



Scheme 3. Prodrug derivatization of KGP94 (**18**) to form water-soluble salt KGP420 (**31**).

Successful completion of the phosphate prodrug was accomplished through phosphorylation of 3-bromophenyl-3-hydroxyphenyl methanone **27** with dibenzyl chlorophosphate (prepared *in situ*)[55] to afford the corresponding dibenzyl phosphate ester **28** (Scheme 3). Subsequent deprotection of the benzyl groups with 33% HBr in AcOH generated phosphoric acid ester **29**. Successful completion of the synthesis of the

phosphate salt of benzophenone thiosemicarbazone KGP420 (**31**) was accomplished by the condensation of phosphoric acid ester **29** with thiosemicarbazide to afford benzophenone thiosemicarbazone **30**, which upon reaction with sodium carbonate generated the desired disodium phosphate salt KGP420 (**31**). Interestingly, KGP420 (**31**) represents the second known example of a reported scaffold incorporating both a thiosemicarbazone and phosphate moiety.[56]

One challenge inherent to the synthesis of thiosemicarbazone based inhibitors is the propensity for isomerization about the imine bond.[57–59] As a notable example, 2-formylpyridine-4',4'-dimethyl thiosemicarbazone, isolated in the *Z* configuration, isomerized in solution generating varying *Z*/*E* equilibrium isomeric ratios which were dependent on the capability of the particular solvent to disrupt the intramolecular hydrogen bonding interaction between the pyridine nitrogen and N-H hydrogen of the thiosemicarbazone.[60] The benzophenone thiosemicarbazone analogues reported herein, except KGP420 (**31**) (60:40 isomeric mixture in D_2O), exist predominately in the *E* configuration in solution (Figure 4). Previous molecular modeling of cathepsin L and KGP94 showed the thiosemicarbazone moiety oriented in the *E* configuration[47] which correlates with the orientation of the major isomer found in solution.

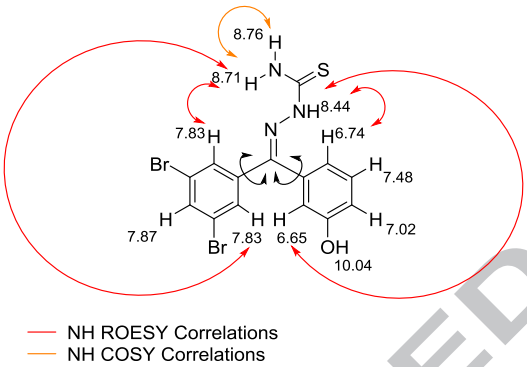


Figure 4. ROESY and COSY correlations for analogue **22** as a representative example of the major isomer observed in DMSO-d_6.

Isomerization of KGP94 (**18**) and benzophenone thiosemicarbazone analogues in DMSO-d_6 occurred over a period of time (Table 1). Analogue **26** isomerized the least with only 9% of the minor isomer present after standing in DMSO-d_6 for one week. Increasing the number of *m*-bromo substituents on the ring *trans* to the –NH of the thiosemicarbazone moiety of the major isomer, and increasing the number of *m*-hydroxy substituents on the ring *cis* to the –NH of the thiosemicarbazone moiety in the major isomer led to an increase in the concentration of *E*-isomer present at equilibrium.

Table 1. Isomerization of benzophenone thiosemicarbazone analogues in DMSO-d_6

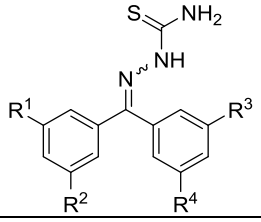
	Percent *Z* isomer present [a]		
Cmpd	0 hours	48 hours	1 week
18	3%	23%	23%
22	1.5%	17%	18%
23	4%	12%	20%
24	11%	13%	13%
25	2%	10%	14%
26	0.2%	8%	9%

[a] Isomerization of KGP94 and analogues were monitored by ^{1}H NMR in DMSO-d_6 as solvent.

The isomer ratio was affected to the greatest degree by the replacement of hydrogen with a hydroxyl substituent in the *meta* position as demonstrated by the decrease of *Z*-isomer present at equilibrium as exemplified by the comparison of analogues **18** (23% *Z*-isomer) and **24** (13% *Z*-isomer) as well as analogues **22** (18% *Z*-isomer) and **26** (9% *Z*-isomer). The replacement of a hydrogen with a bromo substituent in the *meta* position on the ring trans to the –NH of the thiosemicarbazone moiety of the major isomer led to a decrease of *Z*-isomer present at equilibrium as demonstrated by the comparison of analogues **18** and **22**, **23** and **25**, **24** and **26**. Although this is a limited data set, it is interesting to note correlations between isomer ratios and substituent effects. For further analysis see supplementary material.

(3,5-Dibromophenyl)-(3-hydroxyphenyl) ketone thiosemicarbazone (**22**) exhibited comparable activity to KGP94 (**18**)[47] against cathepsin L with an IC_{50} value of 202 nM (Table 2). Using Morrison's equation for tight binding (or covalent, reversible) inhibitors we obtained a K_i^{app} of 8.4 nM (5 min pre-incubation) compared to a value of 3.7 nM for compound **18**. The progress curves for compound **22** demonstrated the time dependence of inhibition (see supplementary material). With cathepsin L inhibition of 40% - 52% at 10 μM, activity of the symmetrical, dimethylresorcinol, and resorcinol thiosemicarbazone analogues **20, 23, 25, 26** bordered the internal cutoff threshold in our laboratory (percent inhibition ≤ 50% at 10 μM). While the presence of one *m*-hydroxyl group on the aromatic ring opposing the 3-bromophenyl substituent in the thiosemicarbazone analogues was important for activity against cathepsin L, for this group of compounds, the presence of two *m*-hydroxyl or two *m*-dimethoxy substituents diminished inhibitory activity against cathepsin L. KGP94 (**18**) did not display significant activity against other proteases such as matrix metalloprotease MMP-9 or the cysteine protease caspase-3 (See supplementary material).

Table 2. Inhibitory activity of benzophenone thiosemicarbazone analogues.



					IC_{50} [a] Values (nM)	
Cmpd	R^1	R^2	R^3	R^4	CatL	CatB
18	H	Br	H	OH	189[b]	>10000[b]
20	Br	OH	Br	OH	>10000	>10000
22	Br[c]	Br[c]	H[c]	OH[c]	202	>10000
23	H	Br	OCH_3	OCH_3	>10000	>10000
24	H	Br	OH	OH	~10000	>10000
25	Br	Br	OCH_3	OCH_3	>10000	>10000
26	Br	Br	OH	OH	>10000	>10000
31	H	Br	H	O-P(O)$O_2$$Na_2$	>10000	ND[d]

[a] These values are averages of a minimum of a triplicate of experiments. Each assay utilized 2% DMSO with a 5 min pre-incubation period. Standard error values can be found in the supplementary material. [b] Previously reported by us.[47] [c] Assigned R groups in Table 2 do not correspond to assigned R groups in Scheme 2. R groups were arranged in this manner to provide clarity for the SAR study. [d] Not Determined.

The phosphate prodrug KGP420 (**31**) demonstrated aqueous solubility greater than 400 mg per mL in comparison to the anti-cancer agent KGP94 (**18**) which demonstrated aqueous solubility less than 0.67 mg per mL (see supplementary material). The stability of phosphate prodrug KGP420 (**31**) was evaluated in aqueous solution. KGP420 (**31**) underwent very minor spontaneous hydrolysis over 48 h incubation at 37 °C in 10 mM glycine buffer solution (pH 8.6) without alkaline phosphatase (ALP) (Figure 5A). Additionally, it was not hydrolyzed when stored in water at 4 °C for one week. Enzymatic cleavage of prodrug KGP420 (**31**) occurred with nearly 100% conversion to the anticipated parent drug KGP94 (**18**) when treated with 1 unit of ALP over the course of 48 hours (Figure 5B). Enzymatic cleavage of KGP420 (**31**) yielded the active product KGP94 (**18**) which inhibited cathepsin L by 88% at 10 µM.

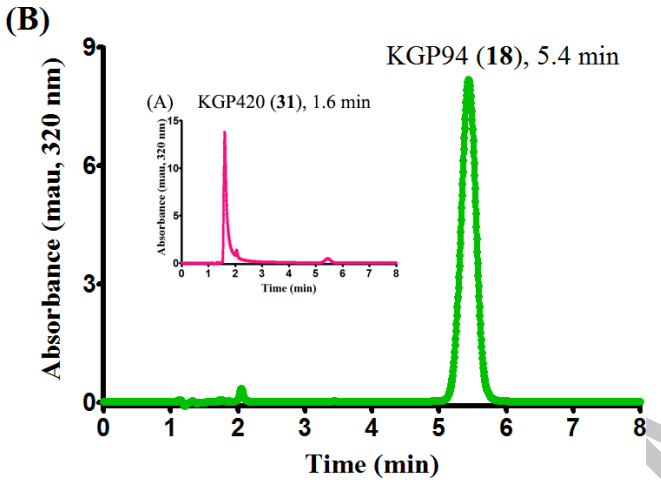


Figure 5. Cleavage of KGP420 (**31**) with alkaline phosphatase (ALP) analyzed by HPLC. (A) Control, **31** (t_R = 1.6 min) was incubated for 48 h without ALP; (B) **31** was treated with 1 unit of ALP for 48 h, with a single product peak **18** (t_R = 5.4 min) observed.

KGP94 (**18**) and the corresponding prodrug KGP420 (**31**) were evaluated for cytotoxicity toward normal primary cells. HUVECs were used a model for normal primary cells. Both KGP94 (**18**) and KGP420 (**31**) did not exhibit significant cytotoxicity against HUVECs especially compared to FDA approved cancer therapeutics doxorubicin and paclitaxel (Table 3). Aggressive cell migration is associated with a metastatic phenotype, and compounds **22, 18**, and **31** significantly inhibited the migration of MDA-MB-231 breast cancer cells compared to the vehicle treated control cells in a scratch assay (see supplementary material).

Table 3. Cytotoxicity against HUVECs

Compound	Doxorubicin	Paclitaxel	KGP94 (**18**)	KGP420 (**31**)
Cytotoxicity GI_{50} (µM)	0.0268[a]	0.00148[a]	26.9	20.2

[a] Previously reported[51]
[b] For additional data see reference[47]

Improved methodology resulted in an alternative synthesis of KGP94, which successfully circumvented unwanted byproduct formation. Benzophenone thiosemicarbazone-based analogues which exhibited a lower threshold for isomerization compared to KGP94 were prepared and evaluated for inhibitory activity against cathepsin L. The most potent of these, [(3,5-dibromophenyl)(3-hydroxyphenyl) ketone] thiosemicarbazone exhibited comparable activity to KGP94 with an IC_{50} value of 202 nM. Advancement of the pre-clinical candidate, KGP94, through phosphate prodrug derivatization to generate KGP420 resulted in a desirable water soluble analogue. In vitro studies demonstrated that KGP420 was hydrolyzed to the parent compound, KGP94, in the presence of alkaline phosphatase. Additionally, KGP420 favorably displayed low cytotoxicity to HUVECs, which were used as a model for normal cells. The in vitro cleavage studies coupled with the desirable property of low cytotoxicity positions KGP420 for future in vivo studies and pre-clinical evaluation as a prodrug.

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Supplementary Data

Supplementary data (Experimental details regarding synthesis, characterization (^{1}H NMR, ^{13}C NMR, ^{31}P NMR, COSY, ROESY, HRMS, HPLC, and X-ray data (KGP94 (**18**) CDCC deposition number: 1445273), and biological assays) associated with this article can be found, in the online version, at