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Mutual prodrugs containing bio-cleavable and drug releasable disulfide linkers

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This article is dedicated to Professors H. Junjappa and H. Ila for their outstanding contribution to the chemistry of Sulfur.

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ABSTRACT

We report herein the design and synthesis of several representative examples of novel mutual prodrugs containing nine distinct types of self-immolative drug-releasable disulfide linkers with urethane, ester, carbonate, or imide linkages between the linker and any two amine/amide/urea (primary or secondary) or carboxyl or hydroxyl (including phenolic)-containing drugs. We also report drug release profiles of a few representative mutual prodrugs in biological fluids such as simulated gastric fluid and human plasma. We also propose plausible mechanisms of drug release from these mutual prodrugs. We have also conducted a few mechanistic studies based on suggested sulfhydryl-assisted cleavage of mutual prodrugs and characterized a few important metabolites to give support to the proposed mechanism of drug release from the reported mutual prodrugs.

1. Introduction

Combination therapy or polytherapy involving two or more therapeutic agents has been in use initially for the treatment of diseases such as AIDS/HIV, cancer, leprosy, malaria and tuberculosis and its use is now extended further to the treatment of many other disorders such as cardiovascular, cardiometabolic, gastrointestinal and musculoskeletal. The rationale for using such combination pill or polypill is to harness the potential synergistic or additive effects of constituent drugs leading to better treatment outcomes. However, it could be a difficult task sometimes to formulate a combination pill or polypill containing two or more active pharmaceutical ingredients with different physicochemical properties and such a hurdle could be overcome by covalently coupling the constituent drugs in a combination therapy to yield a molecular entity called 'mutual prodrug' or 'codrug' [1–4]. Moreover, there could be potential advantages such as improved delivery and pharmacokinetic properties in delivering the co-administered agents as a single chemical entity.

By definition, a mutual prodrug consists of two pharmacologically active agents coupled together so that each acts as a promoiety for the other agent and *vice versa* [1]. Constituent drugs in a mutual prodrug can be covalently coupled either directly or via a suitable linker. Two classic examples of approved mutual prodrugs are estramustine phosphate (1a) [5] and sultamicillin (1b) [6] (Fig. S1, Supporting Information). Estramustine is used in the treatment of prostate cancer and it is composed of an antiandrogenic 17α-estradiol and an alkylating agent normustard [$HN(NCH_2CH_2Cl)_2$] linked via a carbamate linkage. The phosphate group on the estradiol moiety was introduced to improve its water solubility. Both the carbamate and phosphate groups in 1a are hydrolyzed *in vivo* to release the constituent drugs. Sultamicillin is a mutual prodrug with synergistic action and is composed of an irreversible

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-lactamase inhibitor sulbactam and the antibiotic ampicillin connected via a double ester linkage which can be hydrolyzed *in vivo* to release both the drugs simultaneously.

Ideally, a mutual prodrug design must meet the following criteria: (a) the mutual prodrug itself should be inactive or less active than the parent drug(s); (b) both the constituent drugs must possess at least one derivatizable functional group such as an amine, carboxyl, hydroxyl [7]; (c) the linker should be bio-cleavable (either enzymatically or non-enzymatically); (d) the linker or the fragments released from the linker should be nontoxic; (e) release of parent drugs from the mutual prodrug must occur rapidly to ensure therapeutically effective levels of both the constituent drug(s) at the site of action [2].

As an extension of our mission to exploit the bio-cleavable and drug-releasable disulfide linker technology [8–11], we report herein the design and synthesis of several representative examples of novel mutual prodrugs containing nine distinct types of bio-cleavable, self-immolative and drug-releasable disulfide linkers (Fig. 1). We also report a few mechanistic studies to determine the drug release profiles of a few representative mutual prodrugs in biologically relevant fluids such as simulated gastric fluid and plasma and propose plausible mechanisms of drug release from these mutual prodrugs.

2. Results and discussion

In this study, in addition to exploiting our disulfide linker technology [8–11], we also aimed at identifying pairs of representative drugs with functional group(s) that are essential to generate appropriate bio-cleavable covalent linkages between the constituent drug(s) and the disulfide linker. Consequently, some of the mutual prodrugs synthesized in this study may not be of any therapeutic value, but they do serve as good models to subsequently design potentially useful mutual prodrugs of appropriate pairs of drugs that would offer the desired additive and/or synergistic effects.

2.1. Design of mutual prodrugs

The most important feature in our design of mutual prodrugs **2**–**10** (Fig. 1) is the presence of an appropriately positioned disulfide group that is at β-position to bio-labile linkages connecting constituent drugs at both sides of the linker. While drug release from ester-bearing mutual prodrugs could occur *in vivo via* enzymatic hydrolysis, we logically propose here a plausible sulfhydryl-assisted cleavage of the mutual prodrugs containing carbonate, ester, urethane and imide linkages as shown in Scheme 1. For brevity, we present here a generic form of mutual prodrug that depicts a drug-releasable self-immolative disulfide linker and all four types of linkages [i.e., carbonate, ester, imide and urethane] between the constituent drugs and the linker. We thus logically suggest that an intracellular sulfhydryl-containing species such as cysteine or glutathione (RSH or its thiolate anion RS^- at biological pH) would attack the disulfide bond in the mutual prodrug to give the fragments [**M1**] and [**M2**]. The thiolate anion in [**M1**] would trigger cyclization by attacking the proximal carbonyl group of carbonate/urethane/imide linkage (route a) to release the first drug (D^1-XH) along with the cyclic metabolite ethylene monothiolcarbonate [**M3**] [12]. Alternatively, the metabolite **M1** could undergo cyclization followed by decarboxylation (route b) to give thiirane (**M4**) and free drug (D^1-XH). The ester-bearing intermediate metabolite [**M2**] could undergo sulfhydryl-assisted cleavage (route a) to give thiirane (**M4**) and free drug (D^2-CO_2H). Alternatively, the metabolite **M2** could undergo hydrolysis by plasma esterases (route b) to release the second drug (D^2-CO_2H) and the

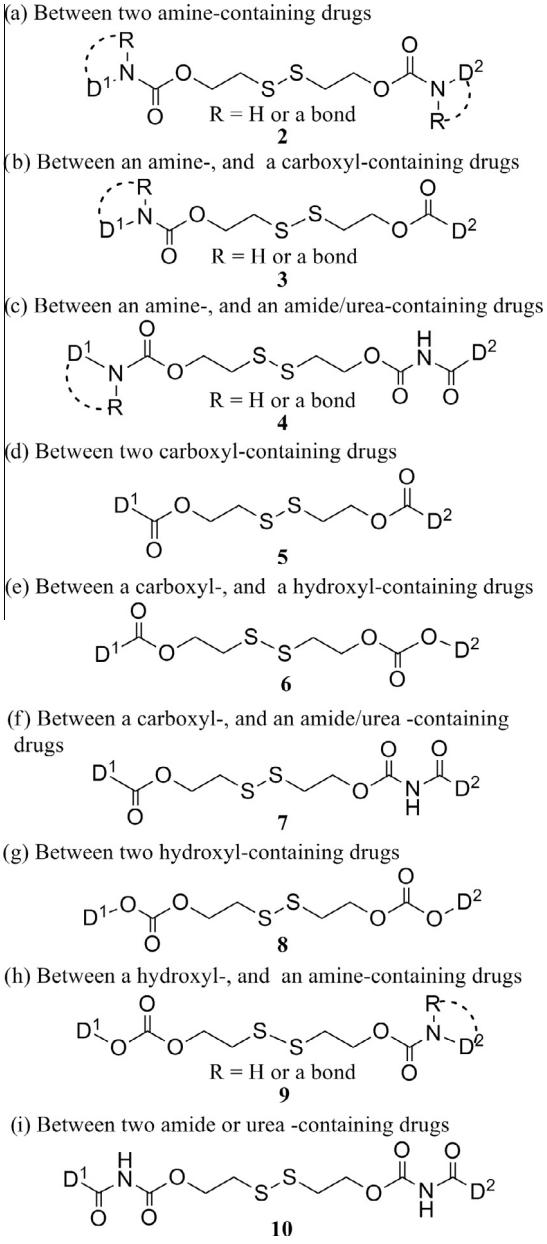
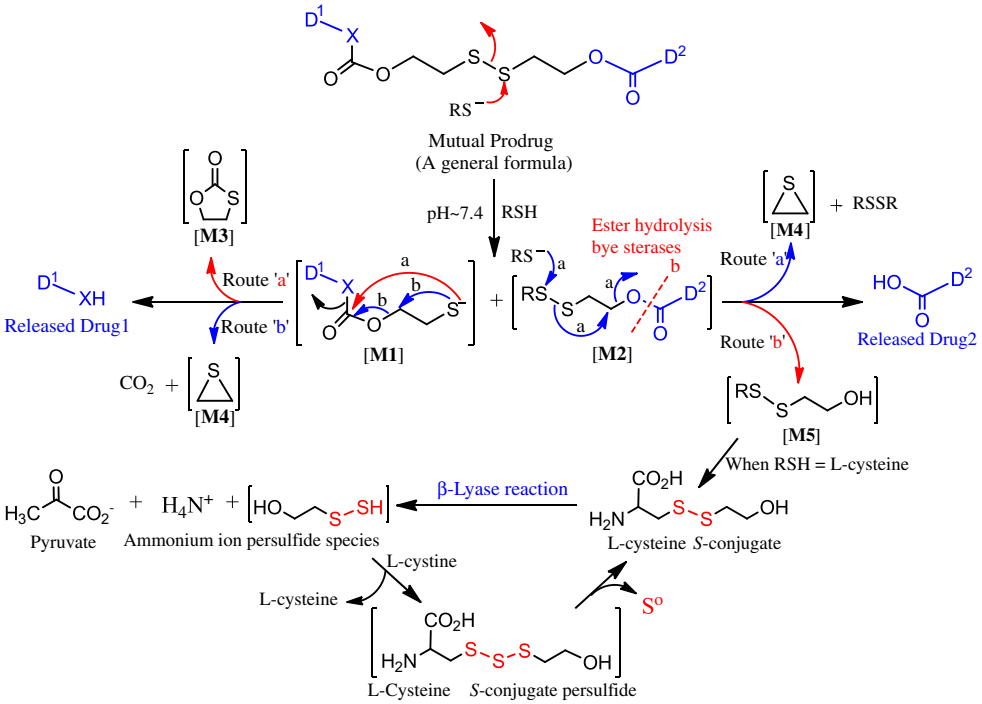


Fig. 1. Mutual prodrugs containing a bio-labile disulfide linker with urethane, ester, carbonate or imide linkages between the linker and two drugs with appropriate derivatizable amide/urea or amine (primary or secondary) or carboxyl or hydroxyl (including phenol) functional groups.

RS-S-containing conjugate [**M5**] that could be excreted. If RSH involved is L-cysteine, then the metabolite **M5** would be a cysteine-*S*-conjugate and such cysteine *S*-conjugates could possibly undergo β-lyase catalyzed breakdown into pyruvate, ammonium ion and a reactive persulfide species [13]. The persulfide species thus formed could rapidly react with L-cystine to give L-cysteine and a reactive cysteine-*S*-conjugate persulfide species which could release elemental sulfur to regenerate the L-cysteine *S*-conjugate. This cycle could probably continue until all relevant species are excreted or metabolized into some biologically inert species.

It is also plausible to think here that the cysteine residues present in intracellular proteins could as well participate in such disulfide exchange reactions with the exogenous disulfide-containing compounds resulting in modified proteins which could lead to some unwarranted biological response. However, by having intracellular concentrations of cysteine and glutathione reduced (GSH)



Wherein, D^1-XH = Drug1, where, X=O, NR (R = H or a bond linked to the D^1 residue), CONH, NCONH; D^2-CO_2H = Carboxyl-containing Drug2; RSH = Cellular Cysteine or Glutathione reduced; RSSR = Cystine or Glutathione oxidized.

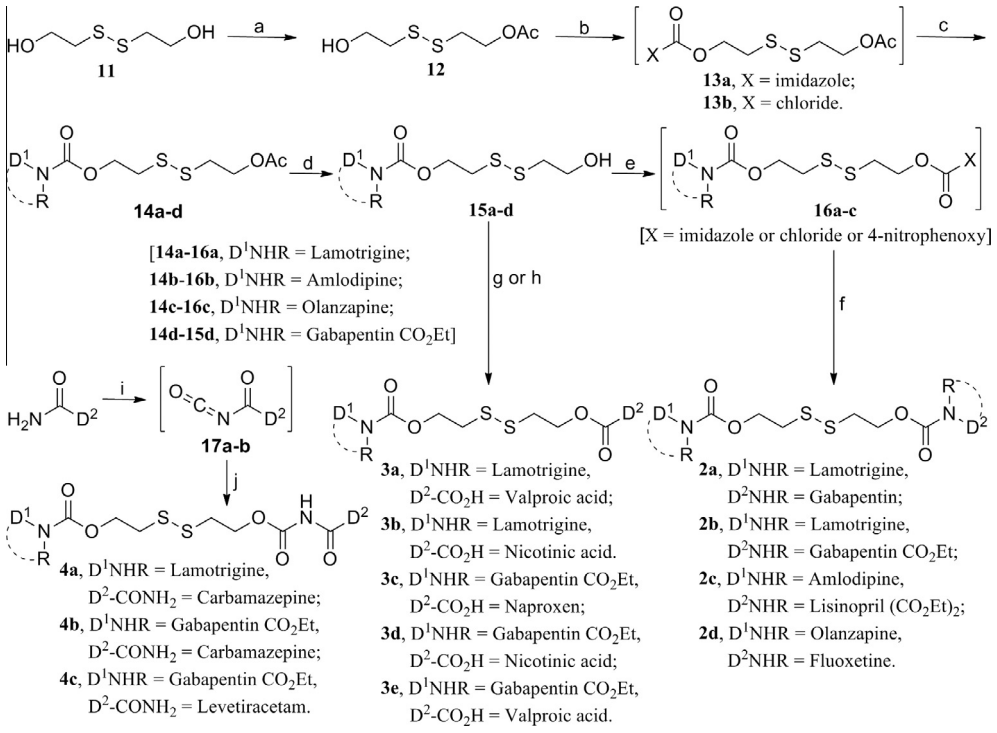
Scheme 1. Plausible mechanisms of drug release by sulfhydryl-assisted and/or esterase-assisted cleavage of the reported mutual prodrugs *in vivo*.

in μM and mM range, respectively, cells maintain appropriate REDOX balance. It is also known that GSH is a substrate for glutathione transferases (GSTs), which are considered as a major family of detoxification enzymes. Thus, cells can maintain sufficient quantities of thiolate anions (i.e., Cys-S^- or GS^-) when it is necessary to neutralize any exogenous disulfide-containing species entering the cells. This could minimize the possibility of protein cysteine residues from participating in such undesirable disulfide exchange reactions.

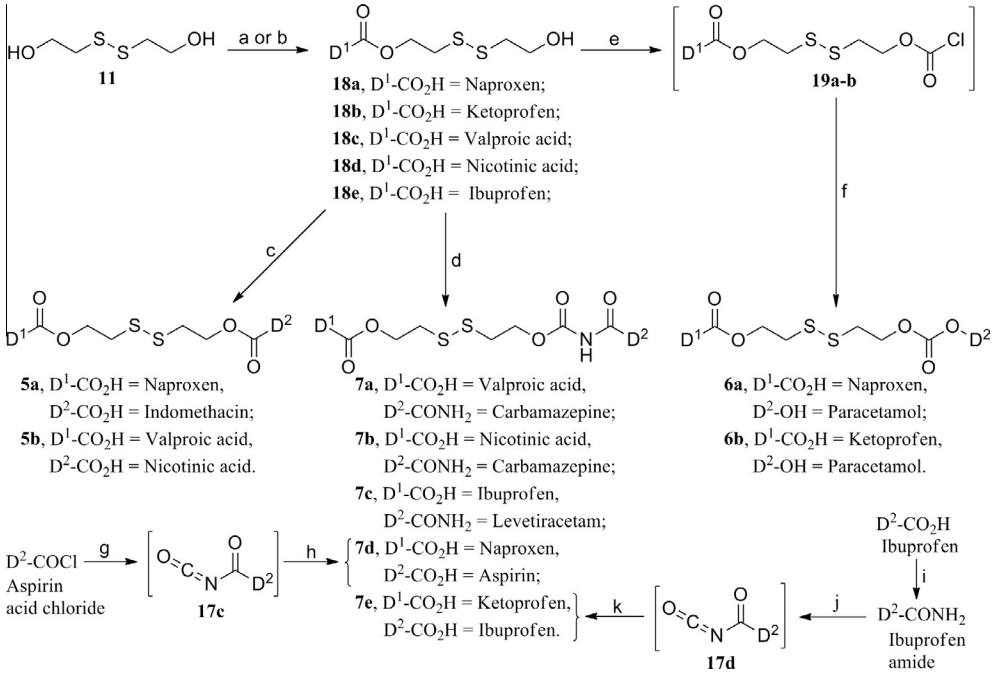
2.2. Synthesis of mutual prodrugs

Synthesis of mutual prodrugs **2a–d**, **3a–e** and **4a–c** was performed as depicted in Scheme 2. Firstly, the commercially available 2,2′-dithiodiethanol (**11**) was treated with acetic anhydride in presence of pyridine to yield the mono-acetyl derivative **12**. Treatment of **12** with 1,1′-carbonyldiimidazole (CDI) or diphosgene yielded the corresponding imidazolide (**13a**) or formyl chloride (**13b**) intermediates which were then treated with appropriate amino-containing drugs (i.e., lamotrigine or amlodipine or olanzapine or gabapentin ethyl ester) in presence of a suitable base such as triethylamine or diisopropylethylamine to yield the corresponding intermediates **14a–d**. The intermediates **14a–d** were deacetylated to give the corresponding intermediate alcohols **15a–d** which were then treated with a phosgene equivalent (i.e., CDI or diphosgene or 4-nitrophenyl chloroformate) to yield the corresponding imidazolide (**16a**) or formyl chloride (**16b**) or 4-nitrophenoxy (**16c**) intermediates, respectively. Treatment of intermediates **16a–c** with appropriate amino-containing drugs (i.e., gabapentin or gabapentin ethyl ester or lisinopril diethyl diester or fluoxetine) in presence of a suitable base such as diisopropylethylamine or triethylamine or aqueous sodium bicarbonate yielded the corresponding mutual prodrugs **2a–d**. Treatment of alcohol intermediates **15a–d** with appropriate carboxyl-containing drugs (i.e., valproic acid or nicotinic acid or naproxen) either as free acids in presence of DCC, DMAP and triethylamine or as acid chlorides in presence of triethylamine, yielded the corresponding mutual prodrugs **3a–e**. Treatment of urea or carbamate-containing drugs (i.e., carbamazepine or levetiracetam) with oxalyl chloride under refluxing condition yielded the corresponding acyl isocyanates **17a–b** which were then treated with appropriate intermediate alcohol **15a** or **15d** to yield the corresponding mutual prodrugs **4a–c**.

The mutual prodrugs **5a–b**, **6a–b** and **7a–e** were synthesized as shown in Scheme 3. Thus, treatment of the diol **11** with appropriate carboxyl-containing drugs (i.e., naproxen or ketoprofen or valproic acid or nicotinic acid or ibuprofen or aspirin) either as free acids in presence of DCC, DMAP and triethylamine or as acid chlorides in presence of triethylamine yielded the corresponding intermediate alcohols **18a–f**. The intermediate alcohols **18a** and **18c** were treated with appropriate carboxyl-containing drugs (i.e., indomethacin and nicotinic acid, respectively) as acid chlorides in presence of triethylamine to yield the corresponding mutual prodrugs **5a–b**. The intermediates **18c–e** were treated with the appropriate acyl isocyanates **17a** or **17b** (Scheme 2) to yield the corresponding mutual prodrugs **7a–c**. The intermediates **18a–b** were treated with phosgene in presence of triethylamine to yield the corresponding formyl chlorides **19a–b**, which were then treated with paracetamol to yield the corresponding mutual prodrugs **6a–b**. Aspirin acid chloride, which was freshly made by treating aspirin with oxalyl chloride in presence of catalytic amount of DMF, was treated with silver cyanate to yield the corresponding acyl isocyanate **17c**, which was then reacted with the intermediate alcohol **18a** to yield the mutual prodrug **7d**. Ibuprofen acid chloride, which was freshly made by treating ibuprofen with oxalyl chloride in presence of catalytic amount of DMF, was treated with ammonia to yield ibuprofen amide. This amide was treated with oxalyl chloride under reflux conditions to yield the acyl isocyanate



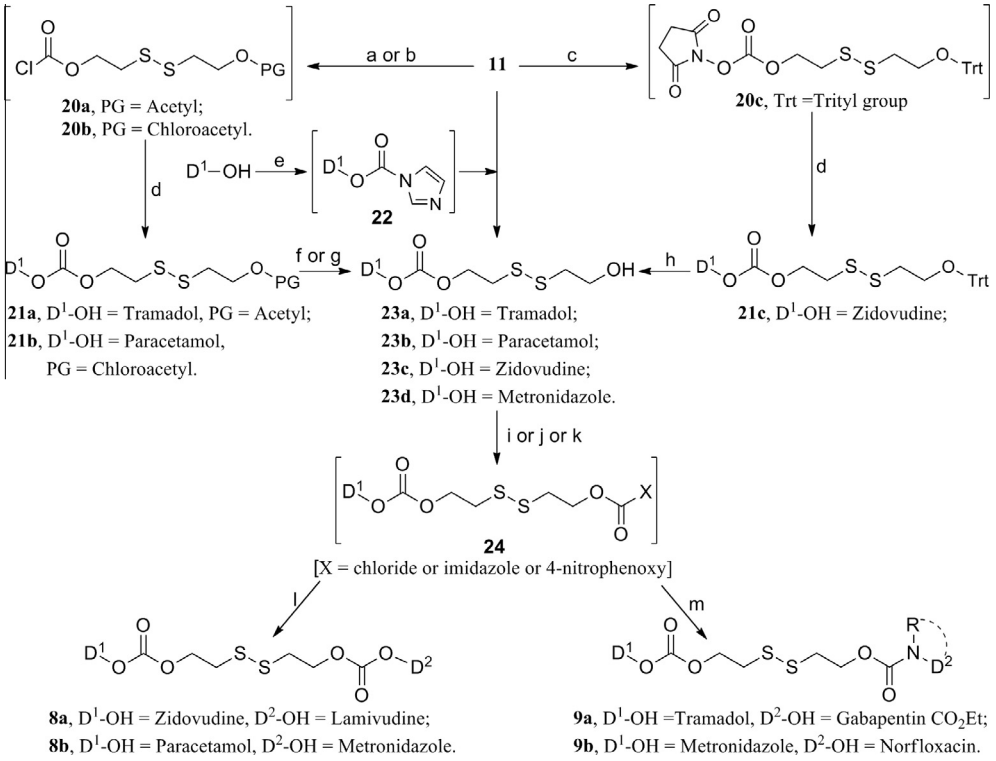
Scheme 2. Synthesis of mutual prodrugs **2a–d**, **3a–e** and **4a–c**. Reagents and conditions: (a) Ac_2O, pyridine, 42%; (b) diphosgene, CH_2Cl_2 (or) CDI, THF; (c) D^1NHR, DIPEA or TEA, 24–79%; (d) 1N K_2CO_3, MeOH:THF (3:1), 60–83%; (e) diphosgene, CH_2Cl_2 (or) CDI, THF/DMF/CH_2Cl_2 or 4-nitrophenyl chloroformate, CH_2Cl_2; (f) D^2NHR, DIPEA or TEA or $NaHCO_3$ aq., 12–93%; (g) D^2-CO2H, DCC, DMAP, TEA, CH_2Cl_2, 20–79%; (h) D^2-COCl, TEA, CH_2Cl_2, 35–90%; (i) $(COCl)_2$, DCE or CH_2Cl_2, reflux; (j) **15a** or **15d**, CH_2Cl_2, RT, 38–50%.



Scheme 3. Synthesis of mutual prodrugs **5a–b**, **6a–b** and **7a–e**. Reagents and conditions: (a) D^1-CO_2H, DCC, DMAP, TEA, THF, 35%; (b) [D^1-COCl] (freshly prepared by oxalyl chloride and DMF method), TEA, THF, 69–77%; (c) [D^2-COCl] (in case of indomethacin, it was freshly prepared by oxalyl chloride and DMF method), TEA, THF, 89–95%; (d) **17** (Scheme 2), DCE or CH_2Cl_2, 12 h, 42–73%; (e) phosgene, TEA, CH_2Cl_2; (f) D^2-OH (paracetamol), TEA, CH_2Cl_2, 67%; (g) AgNCO, benzene, reflux (85–90 °C), 2 h; (h) **18a**, CH_2Cl_2, RT, 16 h, 25%; (i) (i) oxalyl chloride, DMF (2 drops), CH_2Cl_2, 4 h; (ii) 0 °C, NH_3 gas, 30 min, quantitative; (j) $(COCl)_2$, DCE, reflux; (k) **18b**, CH_2Cl_2, RT, 16 h, 47%.

intermediate **17d**, which was then treated with the intermediate alcohol **18b** to afford the mutual prodrug **7e**.

Synthesis of mutual prodrugs **8a–b** and **9a–b** was carried out as shown in Scheme 4. Thus, the diol **11** was treated with acetic anhydride in presence of pyridine or chloroacetyl chloride in presence of triethylamine to yield the corresponding mono-acetyl or mono-chloroacetyl derivatives, which were then treated with diphosgene in presence of triethylamine to yield the corresponding formyl chlorides **20a** and **20b**. Treatment of **20a** and **20b** with hydroxyl-containing drugs tramadol and paracetamol, respectively,



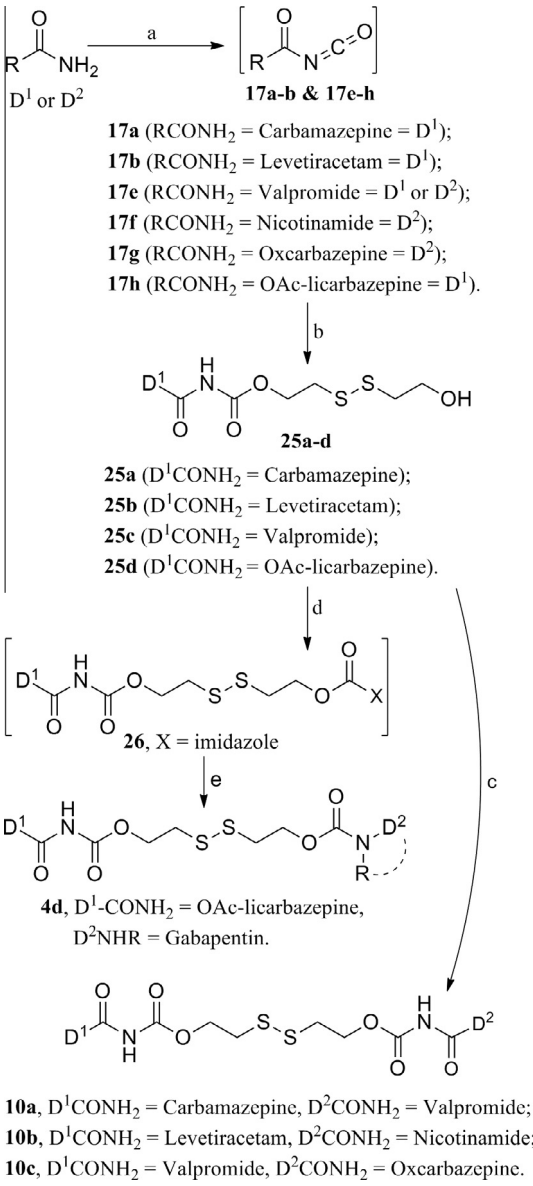
Scheme 4. Synthesis of mutual prodrugs **8a–b** and **9a–b**. Reagents and conditions: (a) (i) acetic anhydride, pyridine, 42%; (ii) diphosgene, TEA, CH_2Cl_2; (b) (i) chloroacetyl chloride, TEA, CH_2Cl_2, 61%; (ii) diphosgene, TEA, CH_2Cl_2; (c) (i) [Trt. DMAP]$^+$ Cl^-, CH_2Cl_2, 30%; (ii) DSC, ACN; (d) D^1-OH, DMAP, ACN, 63–80%; (e) CDI, DMF, **11** (wherein, D^1-OH = metronidazole), 43%; (f) for deacetylation: 0.1 N NaOH, THF, 0 °C, 75%; (g) for removal of chloroacetyl group: NH_3, methanol, 0 °C, 90%; (h) 10% TFA in CH_2Cl_2, 67%; (i) diphosgene, TEA, CH_2Cl_2; (j) CDI, DMF; (k) 4-nitrophenyl chloroformate, CH_2Cl_2, 53%; (l) D^2-OH, DMAP, diisopropylethylamine or TEA, CH_2Cl_2, 44–67%; (m) D^2-NHR, diisopropylethylamine, DMF or CH_2Cl_2, 22–51%.

in presence of DMAP yielded the corresponding intermediates **21a** and **21b**. The intermediate **21a** was treated with 0.1 N sodium hydroxide to yield the intermediate alcohol **23a**. Treatment of **21b** with ammonia in methanol yielded the intermediate alcohol **23b**. In an alternative method, treatment of **11** with trityl-DMAP complex yielded the mono-trityl derivative, which was then treated with N,N′-disuccinimidyl carbonate (DSC) to yield the reactive intermediate **20c**, which was further treated with zidovudine in presence of DMAP to afford the intermediate **21c**. The intermediate **21c** was treated with 10% TFA in DCM to yield the intermediate alcohol **23c**. In another alternative method, metronidazole was treated with CDI to yield the reactive intermediate **22**, which was then treated with **11** to afford the intermediate alcohol **23d**. The intermediates **23a–d** were treated with diphosgene or CDI or 4-nitrophenyl chloroformate to yield the reactive intermediates **24** (wherein, X = chloride or imidazole or 4-nitrophenoxy groups), which were then treated with appropriate hydroxyl or amino-containing drugs to yield the corresponding mutual prodrugs. Thus, reaction of appropriate intermediate **24** with lamivudine and metronidazole in presence of DMAP and triethylamine or diisopropylethylamine yielded the mutual prodrugs **8a** and **8b**, respectively. Similarly, treatment of appropriate intermediate **24** with gabapentin ethyl ester and norfloxacin yielded the mutual prodrugs **9a** and **9b**, respectively.

Synthesis of mutual prodrugs **4d** and **10a–c** was performed as shown in Scheme 5. Firstly, urea/amide/carbamate-containing drugs (i.e., carbamazepine, levetiracetam, valpromide, nicotinamide, oxcarbazepine, and O-acetyl licarbazepine) were treated with oxalyl chloride under refluxing conditions to yield the corresponding acyl isocyanate intermediates **17a–b** and **17e–h**. Treatment of the intermediates **17a–b**, **17e** and **17h** (D^1-$CONH_2$ = carbamazepine or levetiracetam or valpromide or OAc-licarbazepine) with the diol **11** yielded the corresponding intermediate alcohol **25a–d**. Reaction of **25d** (D^1-$CONH_2$ = OAc-licarbazepine) with CDI yielded the corresponding reactive intermediate **26**, which was further treated with gabapentin in aqueous bicarbonate to yield the mutual prodrug **4d**. Treatment of intermediates **25a–c** with appropriate acyl isocyanate intermediates **17e–g** (wherein, D^2-$CONH_2$ = valpromide, nicotinamide and oxcarbazepine, respectively) yielded the corresponding mutual prodrugs **10a–c**.

2.3. Metabolic stability of mutual prodrugs in biological fluids

We have selected a few representative mutual prodrugs containing all 4-types of linkages (i.e., carbonate, ester, imide and urethane) and determined their metabolic stability in biologically relevant fluids such as simulated gastric fluid (SGF) and human plasma. Thus, we have incubated bis-urethane-containing prodrug **2d**, ester and carbonate-containing prodrug **6a**, ester and imide-containing prodrugs **7d** and **7e**, and bis-carbonate-containing prodrug **8a** along with metabolically unstable parent drug aspirin in SGF at pH 1.2 and at 37 °C for stated time and determined stability or decomposition by HPLC. The prodrugs **2d** (bis-urethane-containing), **6a** (ester and carbonate-containing), **7e** (ester and imide-containing) and **8a** (bis-carbonate-containing) remained stable in SGF for 180 min (study period). However, aspirin-naproxen mutual prodrug **7d**, which contains an "imide" linkage between aspirin and the linker and an "ester" linkage between naproxen and the linker, degraded quickly to yield salicylic acid (**SA**) and a major metabolite **M6** (Table 1 and Scheme 6). We have determined the structure of the metabolite **M6** as 2-((2-((acetylcarbamoyl)oxy)ethyl)disulfanyl)ethyl 2-(6-methoxynaphthalen-2-yl)propanoate by LCMS. Contrarily, the other ester and imide-containing prodrug **7e**, which contains an "ester" linkage between ketoprofen



Scheme 5. Synthesis of mutual prodrugs **4d** and **10a–c**. Reagents and Conditions: (a) $(COCl)_2$, DCE or CH_2Cl_2, reflux; (b) **11** (Scheme 2), CH_2Cl_2, RT, 25–55%; (c) **25a–c**, **17e–g**, DCE or CH_2Cl_2, RT, 55–75%; (d) **25d**, CDI, THF, RT; (e) D^2NHR, $NaHCO_3$ aq., 50%.

Table 1
Stability of aspirin, **2d**, **6a**, **7d**, **7e** and **8a** in simulated gastric fluid (SGF).[a,b]

Incubation time (min)	Aspirin		**7d**			
	% **Asp**	% **SA**	% **7d**	% **M6**[c]	% **Nap**	% **SA**
0	100	0	93	4	0	2
5	100	0	55	38	0	42
10	100	0	49	46	0	50
15	100	0	46	46	0	54
30	97	3	44	50	0	56
60	94	6	46	46	0	54
120	89	11	36	56	0	64
180	85	15	37	56	0	64
Half-life ($t_{1/2}$)	>180 min		<10 min			

[a] The prodrugs **2d**, **6a**, **7e** and **8a** remained stable for 180 min (study time) in SGF at 37 °C.
[b] Values (Mol%) given are an average from triplicate experiments.
[c] Structure of the metabolite **M6** (t_R = 8.7 min) is confirmed by LCMS as well as synthesis (Scheme 6). **SA** = Salicylic acid; **Asp** = Aspirin; **Nap** = Naproxen.

and the linker and an "imide" linkage between ibuprofen and the linker, showed good stability in SGF under similar conditions. Even the free drug aspirin exhibited considerable stability in SGF by undergoing about 15% decomposition in 180 min.

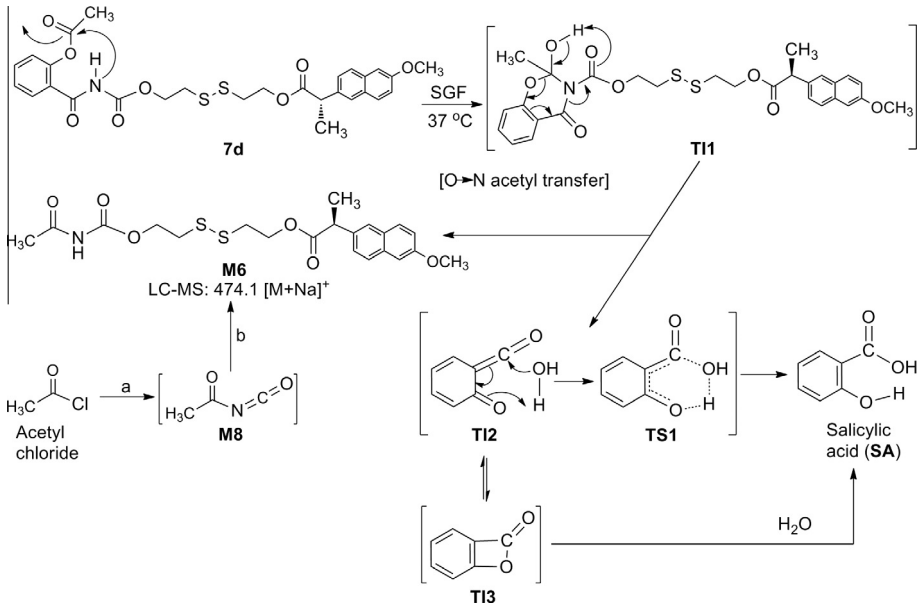
We have proposed a rational mechanism for the formation of the metabolite **M6** and **SA** from the prodrug **7d** as shown in Scheme 6. Thus, the prodrug **7d** could undergo O- to- N-acetyl transfer via a probable transient intermediate TI1, which could break down into the metabolite **M6** and o-oxoketene (**TI2**) [14]. The highly reactive o-oxoketene (**M7A**), which can exist in equilibrium with the lactone form **TI3** [14,15], could rapidly undergo hydration to give a six-membered cyclic transition state intermediate **TS1**, which could then achieve aromatization via rearrangement to yield **SA** [15]. The highly strained lactone form **TI3** (i.e., benzodioxetane] could as well undergo hydration to yield **SA**. We have also confirmed the structure of the major metabolite **M6** by its synthesis as shown in Scheme 6.

Similarly, we have checked metabolic stability of bis-urethane-containing prodrug **2d**, ester and carbonate-containing prodrug **6a**, ester and imide-containing prodrug **7e**, and bis-carbonate-containing prodrug **8a** in human plasma at 37 °C for 60 min and their stability or decomposition was monitored by HPLC (Table 2). As anticipated, the bis-urethane-containing prodrug **2d** showed good stability in human plasma. To our surprise, even the ester and imide-containing prodrug **7e** also exhibited good stability in human plasma during the study period. However, as expected, the other two mutual prodrugs **2d** and **8a**, which carry carbonate linkages, underwent fast decomposition (half-life: <15 min) and released significant amounts of their constituent drugs (Table 2).

2.4. Mechanistic studies

Our proposition of a probable mechanism of drug release from mutual prodrugs containing disulfide linker as shown in Scheme 1 was based on earlier reports by us [8–11], as well as others [12] where the formation of metabolites ethylene monothiolcarbonate (**M3**) and ethylene sulfide (i.e., thiirane, **M4**) was rationally proposed in a similar kind of sulfhydryl-assisted cleavage of other types of prodrugs containing disulfide linkers. However, our efforts to isolate and identify the metabolites **M3** and **M4** were not successful at that time. Now, we have done careful mechanistic studies on sulfhydryl-assisted cleavage of a few representative mutual prodrugs and tried to isolate or identify the said metabolites. First, we were unsuccessful in identifying the molecular masses of the extra metabolites seen in HPLC chromatograms of decomposition mixture at various time points by LCMS as we could not capture their molecular ion peaks which may be due to the low molecular masses of metabolites **M3** and **M4**. As a practical solution, we have synthesized the metabolite ethylene monothiolcarbonate (**M3**) by a known procedure [16] and purchased the thiirane (metabolite **M4**) from commercial sources. We have then conducted sulfhydryl-assisted decomposition studies on a few representative mutual prodrugs and observed formation of the proposed metabolites **M3** and **M4**, which were further confirmed by co-injection with authentic samples of **M3** and **M4** (see Fig. S3, Supporting Information). These results unequivocally provided some credence to the proposed mechanism of drug release from the reported mutual prodrugs.

We have selected bis-carbonate-containing mutual prodrug **8a**, ester and carbonate-containing mutual prodrug **6b**, ester and imide-containing mutual prodrug **7e**, and bis-urethane-containing mutual prodrug **2d** and subjected these compounds to decomposition in the presence of a sulfhydryl-containing species such as dithiothreitol (DTT) [17] under biologically relevant pH (~7.4) and estimated their drug release profiles using HPLC (Table 3).



Scheme 6. A plausible mechanism of cleavage of imide-containing mutual prodrug of aspirin and naproxen **7d** in SGF and synthesis of metabolite **M6** for confirmation of its structure. Reagents and conditions: (a) silver cyanate, toluene, 80 °C, 2 h; (b) **18a** (see Scheme 3), CH_2Cl_2, 50%.

Table 2
Stability of mutual prodrugs **2d**, **6a**, **7e** and **8a** in human plasma.[a,b]

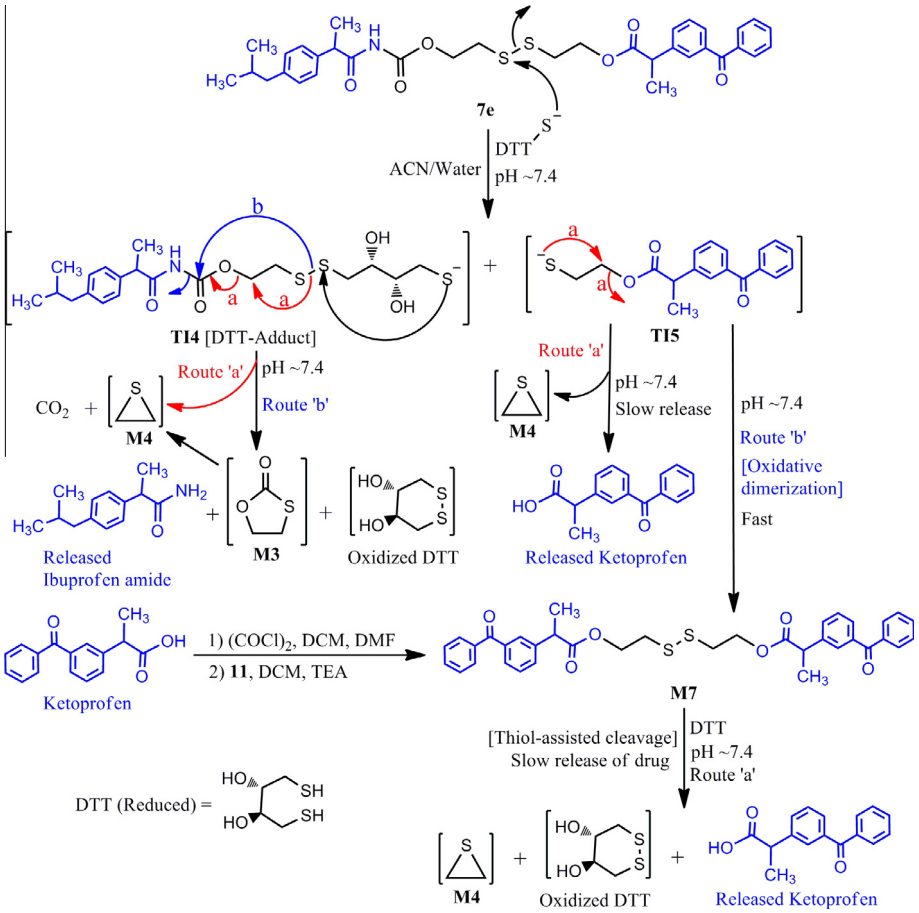
Incubation time (min)	Prodrug **6a**			Prodrug **8a**		
	% **6a**	% **Nap**	% **Par**	% **8a**	% **Lam**	% **Zid**
0	100	0	0	100	0	0
15	22	38	76	0	92	100
30	15	46	88	0	92	100
60	2	68	100	0	94	100
Half-life ($t_{1/2}$)	<15 min			<15 min		

[a] Values (Mol%) given are average of triplicate experiments.
[b] The prodrugs **2d** and **7e** remained stable in human plasma for 60 min (study time) at 37 °C. **Nap** = Naproxen; **Par** = Paracetamol; **Lam** = Lamivudine; **Zid** = Zidovudine.

Thus, the bis-carbonate-containing mutual prodrug **8a** decomposed quickly ($t_{1/2}$ < 5 min) on treatment with DTT at pH ~ 7.4 to release significant amounts of free drugs lamivudine (88%) and zidovudine (60%). The ester and carbonate-containing mutual prodrug **6b** also decomposed quickly ($t_{1/2}$ < 5 min) under similar conditions to release significant amount of paracetamol (70%) and only a slight amount (~4%) of ketoprofen after 60 min. However, there appeared a major metabolite **M7** (24%), which was identified as the ketopropfen dimer by co-injection with an authentic sample of the ketoprofen dimer, which was synthesized as shown in Scheme 7. In this case, as mentioned above, we were successful in the identification of peaks corresponding to the expected metabolites ethylene monothiolcarbonate (**M3**) and ethylene sulfide (i.e., thiirane, **M4**) in the HPLC chromatogram after co-injection of authentic samples of **M3** or **M4** with the decomposition mixture after 2 h of treatment with DTT at pH ~ 7.4 (see Fig. S3, Supporting Information).

Interestingly, when ester and imide-containing mutual prodrug **7e** was treated with DTT at pH ~ 7.4, significant amount (70%) of ibuprofen amide and only 5% of ketoprofen were released after 60 min of reaction time (Table 3). However, there remained 44% of a peak whose UV spectrum is identical to ketoprofen, but differed in retention times. Based on UV spectrum and retention time, we have figured out that the ketoprofen dimer (i.e., metabolite **M7**) and mutual prodrug **7e** shared same retention time! However, they slightly differ in their UV spectrum (see Fig. S4, Supporting Information). As seen in the Spectrum D in Fig. S4, the UV spectrum of the peak supposedly corresponding to the remaining prodrug **7e** shows very slight difference (i.e., having only very slight wedge) to that seen in Spectrum F of Fig. S4, which is corresponding to the pure ketoprofen dimer (**M7**), thereby indicating the presence of higher concentration of the metabolite **M7**. After 30 min of treatment with DTT, the UV spectrum of the peak corresponding to the prodrug **7e** shows a steep curve (see Spectrum E in Fig. S4) which is almost identical to that of the ketoprofen dimer (**M7**) (see Spectrum F in Fig. S4). This UV data therefore confirms that the 70% of prodrug **7e** seen in Table 3 after 30 min treatment of **7e** with DTT is indeed corresponding to the metabolite **M7** and the real half-life of the prodrug **7e** could be <30 min. After 3 h of incubation, HPLC chromatogram showed nearly 90% of ibuprofen amide, about 30% of ketoprofen and about 41% of ketoprofen dimer (**M7**) based on its UV spectrum. After 7 h of incubation, HPLC analysis of the mixture revealed that only 4% of **M7** remained and nearly 70% of ketoprofen and about 80% of ibuprofen amide were released. Based on the identified metabolites, we have logically proposed a plausible mechanism of sulfhydryl-assisted decomposition of the ester and imide-containing mutual prodrug **7e** to release the ibuprofen amide, the metabolites **M3** (i.e., ethylene monothiolcarbonate), **M4** (i.e., thiirane), **M7** (i.e., the ketoprofen dimer) and finally the free drug ketoprofen as shown in Scheme 7. An important observation in this particular study is the formation of ibuprofen amide instead of the free drug ibuprofen from the imide-containing prodrug **7e**. In fact, this result is in line with our earlier observation where an imide-containing NO-flurbiprofen prodrug released flurbiprofen amide *in vitro*, but released only ibuprofen *in vivo* when administered orally to rats [10]. We therefore expect that the imide-containing mutual prodrug **7e** would also release the free drug ibuprofen *in vivo*.

When the bis-urethane mutual prodrug **2d** was similarly treated with DTT at pH ~ 7.4, only 48% of the prodrug remained after 5 min (i.e., $t_{1/2}$ < 5 min) and nearly quantitative amount (~40%) of olanzapine was released. However, there was no release of fluoxetine. However, there appeared three unidentified metabolites, **M8** (20%, its UV matches with olanzapine), **M9** (12%, its UV matches with fluoxetine) and **M10** (20%, its UV matches with fluoxetine).



Scheme 7. A plausible mechanism of drug release via thiol-assisted cleavage of mutual prodrug **7e** and synthesis of the metabolite **M7** for confirmation of its structure and its further thiol-assisted cleavage to release the free drug; DTT = dithiothreitol (reduced).

Table 3
Thiol (DTT)-assisted cleavage of mutual prodrugs **2d**, **6b**, **7e** and **8a**[a].

Incu-bation time (min)	Prodrug **8a**			Prodrug **6b**[b]				Prodrug **7e**			Prodrug **2d**					
	% **8a**	% **Lam**	% **Zido**	% **6b**	% **Keto**	% **Par**	% **M7**[c]	% **7e**[d]	% **Keto**	% **Ibu Am**	% **2d**	% **Ola**	% **Flu**	% **M8**[e]	% **M9**[f]	% **M10**[g]
0	100	0	0	100	0	0	0	100	0	0	100	0	0	0	0	0
5	33	78	56	50	3	27	23	70	1	27	–	–	–	–	–	–
15	–	–	–	–	–	–	–	–	–	–	48	40	0	20	12	20
30	17	82	56	52	3	33	24	70	2	33	–	–	–	–	–	–
60	4	88	60	44	4	70	24	44	5	70	40	52	0	18	6	30
120	–	–	–	10	55	86	13	42	23	86	26	80	–	12	18	33
180	–	–	–	–	–	–	–	41	30	90	–	–	–	–	–	–
240	–	–	–	0	55	76	12	–	–	–	23	100	15	0	69	14
300	–	–	–	0	68	80	1	4	67	76	0	100	48	0	48	2
420	–	–	–	–	–	–	–	4	70	80	–	–	–	–	–	–
20 h	–	–	–	–	–	–	–	–	–	–	0	100	75	0	0	3
Half-life ($t_{1/2}$)	<5 min			<5 min				<60 min			<15 min					

[a] Values (Mol%) given are average of triplicate experiments conducted at pH ~ 7.4.
[b] The metabolites **M3** (t_R = 2.1 min) and **M4** (t_R = 3.13 min) were identified as ethylene monothiolcarbonate and ethylene sulfide (i.e., thiirane), respectively, by co-injection with authentic samples of these compounds (see Fig. S1 in Supporting Information).
[c] The metabolite **M7** is identified as ketoprofen dimer by LCMS and it was further confirmed by synthesis (Scheme 7).
[d] The prodrug **7e** and the metabolite **M7** co-eluted and it is observed by PDA UV absorbance (see Fig. S2 in Supporting Information). Hence, the given Mol% values correspond to both the compounds. Based on UV spectrum, it is observed that the% of **7e** is higher at initial time points and the% of metabolite **M7** is higher at later time points as **7e** degrades slowly to generate ibuprofen amide (**Ibu Am**) and the metabolite **M7**, which eventually undergoes thiol-mediated cleavage to release free ketoprofen (**Keto**) (Scheme 7).
[e] The **M8** (t_R = 5.7 min) is a olanzapine-containing (determined by UV) unidentified metabolite, which degraded with time to release free olanzapine (**Ola**).
[f] The **M9** (t_R = 9.7 min) is a fluoxetine-containing (determined by UV) unidentified metabolite, which degraded with time to release free fluoxetine (**Flu**).
[g] The **M10** (t_R = 11.3 min) is a fluoxetine-containing (determined by UV) unidentified metabolite, which degraded with time to release free fluoxetine. **Nap** = Naproxen; **Par** = Paracetamol; **Lam** = Lamivudine; **Zido** = Zidovudine; – = not done.

Although the mutual prodrug **2d** and the metabolites **M8** and **M10** slowly decomposed with time, the amount of the metabolite **M9** increased during that time. Thus, at 4 h incubation time, the mixture showed 23% of the mutual prodrug **2d**, 100% of olanzapine,

15% of fluoxetine, 0% of **M8**, 69% of **M9** and 14% of **M10**. By the fifth hour, there remained 0% of **2d**, 100% of olanzapine, 48% of fluoxetine, 0% of **M8**, 48% of **M9** and 2% of **M10**. By the twentieth hour, the **M9** also disappeared and the mixture indicated 100% of olanzapine, 75% of fluoxetine and only 3% of **M10** (Table 3).

The above sulfhydryl-assisted decomposition data clearly indicate that the drug release from the reported mutual prodrugs depends not only on the type of linkage between the linker and drug but also on the structural features of the constituent drug. However, the above data would help us to design mutual prodrugs for a particular medical need such as for achieving simultaneous or controlled/sustained or immediate/faster release of constituent drugs *in vivo*.

3. Conclusions

In summary, we have reported a few representative examples of novel mutual prodrugs containing nine distinct types of self-immolative drug-releasable disulfide linkers with urethane, ester, carbonate, or imide linkages between the linker and any two amine/amide/urea (primary or secondary) or carboxyl or hydroxyl (including phenolic)-containing drugs. We have also reported drug release profiles of a few representative mutual prodrugs in biological fluids such as simulated gastric fluid and plasma. We have also proposed plausible mechanisms of drug release from these mutual prodrugs. Based on suggested sulfhydryl-assisted cleavage of mutual prodrugs, we have also conducted a few mechanistic studies and characterized a few important metabolites in support of the proposed mechanism of drug release from the reported mutual prodrugs. We believe that the reported prodrug technology would pave the way for successful design of mutual prodrugs for specific medical needs such as achieving simultaneous or sustained or immediate release of constituent drugs from the mutual prodrugs *in vivo*. Our future work would revolve around application of this prodrug technology to the design, synthesis and evaluation of mutual drugs in a few important therapeutic areas where the use of combination or multi/polypill therapy is a standard treatment option.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2013.06.007.

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[17] In order to achieve quantitative release of the constituent drugs, larger quantities of cysteine or GSH had to be added several times during the in vitro drug release studies as most of the added cysteine or GSH had quickly undergone auto oxidation to their respective disulfide species (Cystine or GSSG) under the reaction conditions. Since it is just a mechanistic study involving a thiolate anion (RS^-), we have used DTT in place of either cysteine or GSH as it could be used in limited quantity to achieve near quantitative release of constituent drugs.