

Design, Synthesis, and Evaluation of Potential Carbamate Prodrugs of 5'-methylthioadenosine (MTA)*

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Abstract

5'-Methylthioadenosine (MTA) is a natural substrate of MTA phosphorylase (MTAP) and is converted to adenine via a salvage pathway for AMP production in normal healthy cells. The lack of MTAP expression in many solid tumors and hematologic malignancies compared to normal healthy cells has been explored in a potential therapeutic strategy to selectively target tumor cells using antimetabolites such as 5-fluorouracil (5-FU) and 6-thioguanine (6-TG) while protecting normal healthy cells with MTA. Herein, a series of carbamate prodrugs, namely the *N*-(alkyloxy)carbonyl-MTA derivatives **2a-f**, was designed, synthesized, and evaluated as potential prodrugs of MTA. All carbamate prodrugs were stable in phosphate buffer, pH 7.4 at 37 °C. In the presence of mouse liver microsomes, the prodrugs were converted to MTA at varying rates with the hexyl and butyl carbamates **2a** and **2b** most readily activated ($t_{1/2}$ of 1.2 and 9.4 h, respectively). The activation was shown to be mediated by carboxyesterases present in mouse liver microsomes.

Introduction

5'-Methylthioadenosine (MTA) is a byproduct formed during polyamine biosynthesis [1] and is a substrate of methylthioadenosine phosphorylase (MTAP) in a process that generates adenine and 5-methylthioribose-1-phosphate [2, 3]. The latter is then converted in several steps to methionine in the methionine salvage pathway. Adenine is converted to AMP by the ubiquitous enzyme adenine phosphoribosyltransferase (APRT) with phosphoribosyl-1-pyrophosphate (PRPP) serving as the donor of the phosphoribosyl group. Although AMP may also be produced in cells by *de novo* purine biosynthesis, MTAP is responsible for the generation of essentially all free adenine in normal healthy cells [4].

Many solid tumors and hematologic malignancies are characterized by a deficiency of MTAP and are unable to salvage adenine from MTA. The difference in the lack of expression of MTAP in tumor cells compared to normal healthy cells has been explored in a therapeutic strategy for selectively killing tumor cells [5, 4]. Antimetabolites such as 5-fluorouracil (5-FU), 6-thioguanine (6-TG) or antifolates disrupt DNA replication and inhibit *de novo* purine synthesis through the release of cytotoxic nucleotides generated via phosphorylation using phosphoribosyl-1-pyrophosphate (PRPP). One major drawback of these cytotoxic nucleotides is that they produce harmful side effects on the growth and proliferation of replicating normal healthy cells. To reduce the cytotoxicity of these chemotherapeutic drugs and thus increase their therapeutic index, administration of MTA or MTA analogs in conjugation with these antimetabolites could prevent damage to healthy MTAP-competent cells [6, 7]. This is because adenine generated in the above adenine salvage pathway by MTAP in healthy cells will block the conversion of 5-FU/6-TG to their cytotoxic nucleotides by competing for the rate-limiting pools of PRPP. Since no adenine is produced in tumor cells due to lack of MTAP, PRPP is present in sufficient levels and the co-administered drug can be readily converted to its toxic metabolite. Thus, a high degree of selectivity can be achieved. Indeed, MTA protection was shown to be quite remarkable *in vitro* with at least 10- to 100- fold changes in IC_{50} values when co-administered with 6-TG, suggesting that higher doses of 6-TG than currently administered could be given before any side effects are seen [3-5, 8, 9]. MTA in high doses has been shown to prevent liver

damage in experimental models [10, 11]. Bertino and coworkers also reported that co-administration of 100 mg/kg MTA protected nude mice from a lethal dose of 5-FU, raising its maximum tolerated dose from 75 to 200 mg/kg [5]. In a clinical study, MTA was dosed daily at 600 mg for one month to 50 volunteers and at 1600 mg daily for one month to another 10 volunteers without reports of any toxicity in either group [12, 13]. These proof of concept preclinical and clinical studies suggest that MTA can produce a beneficial protective effect to healthy cells and thus reduce systemic side effects during cancer chemotherapy using these antimetabolites.

There are several MTA prodrugs disclosed in a US patent by modifying the ribosyl hydroxyl groups in the form of carbamates, phosphates and esters to deliver MTA to protect MTAP-competent cells [14]. Herein, we report the design of new prodrugs of MTA by attaching promoieties to the 6-amino group of adenine for activation by carboxylesterases to release MTA in an effort to overcome the potential stability and bioavailability issues of MTA.

Results And Discussion

Prodrug Design

We focused our design of prodrugs on the 6-NH₂ of MTA that could potentially be activated by a carboxylesterase enzyme [15, 16]. Carboxylesterases are ubiquitously present in most animals including humans, and several types are present in blood, liver and other tissues. The initial design of our MTA prodrugs was based on the activation of the marketed prodrug of 5-FU known as capecitabine. Clinically, capecitabine passes intact through the intestinal mucosa and is converted to its active form, 5-FU, by a 3-step enzymatic activation in liver and tumor tissues. Its activation is initiated by carboxylesterase-mediated hydrolysis of the carbamate moiety to 5'-deoxy-5-fluorocytidine primarily in the liver with a reported half-life of 0.55 to 0.87 hours [17, 18].

We used a similar strategy to design the carbamate or *N*-(alkyloxy)carbonyl-MTA prodrugs **2a-f** as depicted in Figure 1 that can potentially be activated by the liver carboxylesterases for systemic release of MTA. In the process of designing MTA carbamate prodrugs **2a-f**, we varied the alkyl groups of the promoieties from 2 to 6 carbons to discern what effect the alkyl group would have on the rate of cleavage of the carbamate moiety by the liver carboxylesterase. The carbamate functional group can impart chemical stability and since carbamates do not ionize, they can also provide better oral bioavailability for the prodrugs. Upon hydrolysis, the prodrugs would release alcohol and carbamic acid that would be unstable under physiological pH and decompose spontaneously to the parent amine drug (in our case, MTA) and CO₂.

Chemical Synthesis

The syntheses of *N*-(alkyloxy)carbonyl-MTA prodrugs **2a-f** are straightforward and were accomplished in two steps as shown in **Scheme 1**. In step 1, treatment of commercially available MTA with an excess amount (6 eq.) of commercially available alkyl chloroformates in the presence of a base in anhydrous

dichloromethane gave the intermediates **3a-f** with a carbamate on 6-NH₂ of adenine and two carbonates on the two hydroxyl groups of ribose. The carbonates were more easily and selectively hydrolyzed using 0.1 N NaOH to afford the desired prodrugs **2a-f**.

Evaluation of *N*-(alkyloxy)carbonyl-MTA Prodrugs **2a-f**

We first evaluated the stability of the carbamate or *N*-(alkyloxy)carbonyl-MTA prodrugs **2a-f** in 50 mM phosphate buffer, pH 7.4 at 37 °C using an HPLC assay that is capable of separating the prodrugs and the parent molecule MTA on a reverse phase C₁₈ column. As shown in Table 2, all the *N*-(alkyloxy)carbonyl-MTA prodrugs **2a-f** of MTA demonstrated great stability under these conditions with less than 10% change in measured peak area over 72 hours. Then we determined their activation in the presence of mouse or human liver microsomes. In the presence of mouse liver microsomes, the prodrugs were activated at varying rates in the order of hexyl (**2a**) > butyl (**2b**) > propyl (**2e**) > 2-methoxyethyl (**2f**) > isopropyl (**2c**) ≈ ethyl (**2d**) with the hexyl and butyl carbamate prodrugs **2a** and **2b** exhibiting a half-life of 1.2 and 9.4 h, respectively. As shown in Figure 2A and 2B, the activation of prodrugs **2a** and **2b** in the presence of MLM was accompanied with the formation of MTA, parent drug. It is also clear from the MTA decomposition phase shown

in Figure 2B vs 2A that overall degradation of MTA was slower when the prodrug activation was slower in the presence of MLM, suggesting that prodrug **2b** significantly increased the stability of MTA which validates our design principle. However, as shown in Table 2, the activation of the *N*-(alkyloxy)carbonyl-MTA prodrugs in the presence of human liver microsomes (HLM) was much slower with $t_{1/2}$ all greater than 48 h; prodrugs **2a** and **2b** showed about 44% and 19% change, respectively, in the peak area of the prodrugs measured over the 48-h incubation with HLM.

To confirm the type of enzyme involved in the metabolic activation of *N*-(alkyloxy)carbonyl-MTA prodrugs, we performed the activation of **2b** using mouse or human liver microsomes in the presence of 1 mM bis(4-nitrophenyl)-phosphate (BNPP), an inhibitor of carboxyesterases, or 1 mM of nicotinamide adenine dinucleotide phosphate (NADPH), a cofactor needed for oxidative metabolism. As shown in Figure 3, the BNPP completely prevented the activation of **2b** by mouse liver microsomes while NADPH had no effect on the activation of **2b**, confirming that the activation of *N*-(alkyloxy)carbonyl-MTA prodrug **2b** is mediated by a carboxypeptidase(s), not by oxidoreductases, in mouse liver microsomes. Also consistent with the earlier results, the slow activation of **2b** observed in the presence of human liver microsomes is only marginally affected by the addition of BNPP or NADPH.

In summary, we designed, synthesized, and conducted activation studies of carbamate prodrugs of 5'-methylthioadenosine (MTA), in the form of *N*-(alkyloxy)carbonyl-MTA. All of the carbamate prodrugs obtained were stable in phosphate buffer, pH 7.4 at 37 °C. In the presence of mouse liver microsomes, the prodrugs were converted to MTA at varying rates with the hexyl and butyl carbamates **2a** and **2b** most readily activated exhibiting $t_{1/2}$ of 1.2 and 9.4 h, respectively. The activation was shown to be mediated by carboxyesterases present in mouse liver microsomes. The activation of these prodrugs in the presence

of human liver microsomes was much slower with $t_{1/2}$ all greater than 48 h. Consistent with the activation studies in the presence of mouse liver microsomes, the hexyl and butyl carbamates **2a** and **2b** were shown to have the highest level of conversion at 44% and 19%, respectively. Further studies will be needed to fully explore the potential of these carbamate prodrugs.

Experimental Section

All reactions were performed in oven-dried glassware. All solvents used were either ACS reagent grade or HPLC grade. All reactions were performed using anhydrous solvents. Moisture sensitive reactions were performed under N_2 atmosphere. Air sensitive reagents were transferred to a reaction flask using a cannula under N_2 atmosphere. All reactions were monitored by Thin Layer Chromatography (TLC) using 0.25 mm Whatmann precoated silica gel plates. TLC plates were visualized by either UV absorbance or potassium permanganate solution. Purification was done by using automated flash column chromatography using Teledyne ISCO CombiFlash Companion System.

Analytical LC-MS was obtained using Agilent 1200 Series LC system equipped with G6140A Quadruple MS on a C_{18} column. Solvent A was 0.1% formic acid/ H_2O and solvent B was 0.1% formic acid in methanol and the gradient used was 10-90% B in 5 mins at a flow rate of 0.8 ml/min. NMR spectra were recorded on a Bruker Ultrashield 400 MHz at ambient temperature. High Resolution Mass Spectrometry data was obtained at the Center for Integrative Proteomics Research (CIPR), Rutgers University.

General procedure for the synthesis of *N*-(alkyloxy)carbonyl-MTA prodrugs (**2a-f**)

5'-Methylthioadenosine (MTA) was suspended in 10-20 ml anhydrous dichloromethane. 1-Methylimidazole (8 eq.) was added and the suspension was stirred at 0 °C for 5 mins. An alkyl chloroformate (6 eq.) was added dropwise to the cold suspension over 5 mins while maintaining the temperature at 0 °C. The reaction mixture was allowed to warm to room temperature and was stirred for an additional 3 days. The reaction progress was monitored using TLC and LCMS. The reaction mixture was diluted with dichloromethane (20 mL) and washed successively twice each with saturated $NaHCO_3$ (10 ml) and brine (10 mL). The organic phase was separated and dried over Na_2SO_4 and filtered. The solvent was evaporated, and the crude product was purified by column chromatography using methanol and dichloromethane as an eluent to give the intermediate **3a-f** as an oil.

To a solution of intermediate **3a-f** in 5 mL ethanol was added 0.1 N NaOH (1 eq.) and the resulting reaction mixture was stirred at room temperature for 12 hours. On completion of the reaction, 1 N HCl was added to the reaction mixture until the pH was neutral. Dichloromethane (10 mL) was then added to the reaction mixture followed by washing with brine (5 mL). The organic layer was dried over Na_2SO_4 , filtered and the solvent was evaporated. The crude product was purified using column chromatography in methanol in dichloromethane to give final target compounds **2a-f** as white solids.

N-(hexoxycarbonyl)-2',3'-bis(hexylcarbonate)-MTA (**3a**): Yield, 140 mg (61%) obtained from 100 mg (0.336 mmol) of MTA. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 8.64 (s, 1H), 8.50 (s, 1H), 6.34 (d, *J* = 5.3 Hz, 1H), 6.09 (t, *J* = 5.5 Hz, 1H), 5.64 (dd, *J* = 5.7, 4.7 Hz, 1H), 4.46 (td, *J* = 5.9, 4.6 Hz, 1H), 4.26 (t, *J* = 6.7 Hz, 2H), 4.23 – 4.01 (m, 4H), 3.11 – 2.90 (m, 2H), 2.11 (s, 3H), 1.81 – 1.65 (m, 4H), 1.60 (dq, *J* = 8.0, 6.5 Hz, 2H), 1.51 – 1.23 (m, 18H), 0.98 – 0.87 (m, 9H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 154.2, 153.9, 152.2, 152.1, 151.1, 150.0, 142.9, 122.4, 86.3, 81.8, 75.5, 75.4, 68.8, 68.6, 65.7, 35.5, 33.4, 31.3, 31.2, 31.2, 30.9, 28.5, 28.3, 28.2, 27.9, 25.4, 25.3, 25.1, 25.0, 24.7, 22.3, 22.3, 22.2, 22.1, 15.2, 13.1, 13.1, 13.0; LCMS (ESI⁺): 682.3 [M + H]⁺.

N-(hexoxycarbonyl)-MTA (**2a**, LH1201): Yield, 67 mg (77%) obtained from 140 mg (0.205 mmol) of **3a**. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 8.63 (s, 1H), 8.55 (s, 1H), 6.11 (d, *J* = 5.0 Hz, 1H), 4.38 (t, *J* = 4.9 Hz, 1H), 4.29, (t, *J* = 6.2 Hz, 2H), 4.27 (m, 1H), 2.98 (dd, *J* = 14.1, 5.6 Hz, 1H), 2.90 (dd, *J* = 14.1, 6.1 Hz, 1H), 2.14 (s, 3H), 1.85 – 1.69 (m, 2H), 1.48 (s, 2H), 1.42 – 1.31 (m, 4H), 0.95 (t, *J* = 7.2 Hz, 3H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 152.7, 151.3, 150.6, 149.0, 143.1, 121.7, 89.1, 84.3, 73.6, 72.6, 66.1, 36.1, 31.2, 28.5, 25.2, 22.2, 15.2, 13.0; HRMS (ESI⁺) *m/z* calcd for C₁₈H₂₈N₅O₅S [M + H]⁺: 426.1806, found: 426.1816.

N-(butoxycarbonyl)-2',3'-bis(butylcarbonate)-MTA (**3b**): Yield, 130 mg (65%) obtained from 100 mg (0.336 mmol) of MTA. ¹H NMR (400 MHz, CDCl₃) δ (ppm): 9.18 (s, 1H), 8.75 (s, 1H), 8.20 (s, 1H), 6.20 (d, *J* = 5.4 Hz, 1H), 6.04 (t, *J* = 5.4 Hz, 1H), 5.60 – 5.55 (m, 1H), 5.27 (s, 1H), 4.47 (q, *J* = 5.5 Hz, 1H), 4.26 (t, *J* = 6.7 Hz, 2H), 4.22 – 4.01 (m, 4H), 3.05 – 2.85 (m, 2H), 2.10 (s, 3H), 1.73 – 1.52 (m, 6H), 1.47 – 1.26 (m, 6H), 0.97 – 0.83 (m, 9H); LCMS (ESI⁺): 598.2 [M + H]⁺.

N-(butoxycarbonyl)-MTA (**2b**, LH1205): Yield, 60 mg (69%) obtained from 130 mg (0.218 mmol) of **3b**. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 8.63 (s, 1H), 8.55 (s, 1H), 6.11 (d, *J* = 5.0 Hz, 1H), 4.38 (s, 1H), 4.30 (t, *J* = 6.6 Hz, 2H), 4.27 – 4.23 (m, 1H), 2.97 (d, *J* = 5.6 Hz, 1H), 2.92 (d, *J* = 6.1 Hz, 1H), 2.14 (s, 3H), 1.76 (s, 2H), 1.51 (d, *J* = 7.6 Hz, 2H), 1.01 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (125 MHz, Methanol-*d*₄) δ (ppm): 152.5, 151.5, 151.3, 149.6, 142.8, 122.2, 89.1, 84.3, 73.5, 72.7, 65.5, 36.1, 30.6, 18.7, 15.1, 12.6; HRMS (ESI⁺) *m/z* calcd for C₁₆H₂₄N₅O₅S [M + H]⁺: 398.1493, found: 398.1479.

N-(Isopropoxycarbonyl)-2',3'-bis(isopropylcarbonate)-MTA (**3c**): Yield, 190 mg (52%) obtained from 200 mg (0.67 mmol) of MTA. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 8.52 (s, 1H), 8.38 (s, 1H), 6.19 (d, *J* = 5.3 Hz, 1H), 5.96 (t, *J* = 5.5 Hz, 1H), 5.54 – 5.49 (m, 1H), 4.98 (dt, *J* = 12.5, 6.2 Hz, 1H), 4.81 (dd, *J* = 12.5, 6.2 Hz, 1H), 4.67 (dt, *J* = 12.5, 6.2 Hz, 1H), 4.33 (q, *J* = 5.9 Hz, 1H), 2.92 (dd, *J* = 14.3, 5.8 Hz, 1H), 2.86 (dd, *J* = 14.3, 6.1 Hz, 1H), 2.00 (s, 3H), 1.17 (ddd, *J* = 46.6, 20.1, 6.3 Hz, 18H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 155.0, 154.7, 153.6, 153.1, 152.5, 151.4, 144.3, 123.8, 87.7, 83.2, 76.8, 76.6, 74.5, 74.2, 71.0, 37.0, 22.4, 16.7; LCMS (ESI⁺): 556.3 [M + H]⁺.

N-(Isopropoxycarbonyl)-MTA (**2c**, LH1206): Yield, 72 mg (62%) obtained from 170 mg (0.3 mmol) of **3c**. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 10.38 (bs, 1H), 8.67 (s, 1H), 8.64 (s, 1H), 6.01 (d, *J* = 5.8 Hz, 1H), 5.59 (s, 1H), 5.39 (s, 1H), 4.93 (hept, *J* = 6.2 Hz, 1H), 4.83 – 4.77 (m, 1H), 4.19 (s, 1H), 4.11 – 4.05 (m, 1H), 2.91 (dd, *J* = 13.9, 5.9 Hz, 1H), 2.81 (dd, *J* = 13.9, 6.9 Hz, 1H), 2.06 (s, 3H), 1.28 (d, *J* = 6.2 Hz, 6H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 152.5, 150.7, 150.6, 149.6, 141.5, 122.1, 89.7, 84.3, 74.6, 73.1, 70.3, 36.8, 21.9, 21.8, 16.7; HRMS (ESI+) *m/z* calcd for C₁₅H₂₂N₅O₅S [M + H]⁺: 384.1336, found: 384.1350.

N-(Ethoxycarbonyl)-2',3'-bis(ethylcarbonate)-MTA (**3d**): Yield, 197 mg (57%) obtained from 200 mg (0.67 mmol) of MTA. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 8.60 (s, 1H), 8.47 (s, 1H), 6.29 (d, *J* = 5.3 Hz, 1H), 6.04 (t, *J* = 5.4 Hz, 1H), 5.60 (t, *J* = 5.1 Hz, 1H), 4.41 (q, *J* = 5.7 Hz, 1H), 4.28 (q, *J* = 7.1 Hz, 2H), 4.23 – 4.17 (m, 2H), 4.10 (dd, *J* = 14.7, 7.2 Hz, 2H), 2.99 (dd, *J* = 14.4, 5.8 Hz, 1H), 2.93 (dd, *J* = 14.3, 6.2 Hz, 1H), 2.06 (s, 3H), 1.32 (t, *J* = 7.1 Hz, 3H), 1.28 (t, *J* = 7.1 Hz, 3H), 1.19 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 153.4, 153.1, 151.5, 151.4, 150.4, 149.3, 142.2, 121.8, 85.7, 81.1, 74.7, 74.7, 64.0, 63.8, 60.9, 34.8, 14.4, 12.7, 12.5, 12.3; LCMS (ESI⁺): 514.5 [M + H]⁺.

N-(Ethylloxycarbonyl)-MTA (**2d**, LH1207): Yield, 62 mg (57%) obtained from 150 mg (0.29 mmol) of **3d**. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 8.46 (s, 1H), 8.25 (s, 1H), 6.05 (d, *J* = 4.5 Hz, 1H), 4.75 (t, *J* = 4.6 Hz, 1H), 4.41 – 4.37 (m, 1H), 4.30 (q, *J* = 4.9 Hz, 1H), 4.18 (q, *J* = 7.0 Hz, 2H), 2.86 (dd, *J* = 13.8, 5.3 Hz, 1H), 2.78 (dd, *J* = 14.1, 5.3 Hz, 1H), 2.05 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 152.2, 151.7, 151.1, 149.7, 142.6, 122.3, 88.9, 84.1, 73.3, 72.6, 61.5, 36.0, 15.0, 13.2; HRMS (ESI⁺) *m/z* calcd for C₁₄H₂₀N₅O₅S [M + H]⁺: 370.1180. Found: 370.1193.

N-(Propoxycarbonyl)-2',3'-bis(propylcarbonate)-MTA (**3e**): Yield, 175 mg (46%) obtained from 200 mg (0.67 mmol) of MTA. ¹H NMR (400 MHz, Methanol-*d*₄) δ (ppm): 9.16 (s, 1H), 8.77 (s, 1H), 8.20 (s, 1H), 6.21 (d, *J* = 5.3 Hz, 1H), 6.05 (t, *J* = 5.4 Hz, 1H), 5.59 (t, *J* = 5.0 Hz, 1H), 4.49 (q, *J* = 5.3 Hz, 1H), 4.23 (t, *J* = 6.7 Hz, 2H), 4.17 – 4.01 (m, 4H), 3.02 (dd, *J* = 14.3, 5.3 Hz, 1H), 2.94 (dd, *J* = 14.3, 6.1 Hz, 1H), 2.12 (s, 3H), 1.76 – 1.62 (m, 6H), 0.99 – 0.88 (m, 9H); ¹³C NMR (100 MHz, Methanol-*d*₄) δ (ppm): 152.2, 151.7, 151.1, 149.6, 142.6, 122.3, 88.9, 84.1, 73.3, 72.6, 61.5, 36.0, 15.0, 13.2; LCMS (ESI⁺): 556.6 [M+H]⁺.

N-(Propoxycarbonyl)-MTA (**2e**, LH1208): Yield, 45 mg (66%) obtained from 100 mg (0.18 mmol) of **3e**. ¹H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.55 (s, 1H), 8.14 (s, 1H), 5.96 (d, *J* = 5.3 Hz, 1H), 4.69 (t, *J* = 5.3 Hz, 1H), 4.42 – 4.39 (m, 3H), 4.37 – 4.35 (m, 1H), 4.16 (t, *J* = 6.7 Hz, 2H), 2.83 (dd, *J* = 12.9, 5.0 Hz, 1H), 2.78 (dd, *J* = 13.0, 4.6 Hz, 1H), 2.10 (s, 3H), 1.67 (dq, *J* = 14.2, 7.2 Hz, 2H), 0.92 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (100 MHz, Chloroform-*d*) δ (ppm): 152.4, 151.4, 150.6, 149.4, 141.6, 121.9, 89.5, 84.3, 74.7, 73.2, 67.9, 36.8, 22.1, 16.7, 10.3; HRMS (ESI⁺) *m/z* calcd for C₁₅H₂₂N₅O₅S [M + H]⁺: 384.1336, found: 384.1350.

N-(2-Methoxyethoxy)carbonyl-2',3'-bis(2-methoxyethylcarbonate)-MTA (**3f**): Yield, 200 mg (56%) obtained from 200 mg (0.67 mmol) of MTA. ¹H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.79 (s, 1H), 8.69

(s, 1H), 8.09 (s, 1H), 6.09 (d, $J = 5.3$ Hz, 1H), 6.00 (t, $J = 5.3$ Hz, 1H), 5.53 (t, $J = 5.0$ Hz, 1H), 4.39 (q, $J = 5.5$ Hz, 2H), 4.24 (q, $J = 4.5, 4.0$ Hz, 3H), 4.16 (t, $J = 4.6$ Hz, 2H), 3.63 – 3.57 (m, 2H), 3.55 (dd, $J = 6.8, 2.5$ Hz, 2H), 3.50 – 3.45 (m, 2H), 3.31 (d, $J = 6.9$ Hz, 6H), 3.25 (s, 3H), 2.93 (dd, $J = 14.3, 5.4$ Hz, 1H), 2.85 (dd, $J = 14.3, 6.2$ Hz, 1H), 2.03 (s, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 154.1, 153.8, 153.1, 151.0, 150.9, 149.7, 142.2, 122.8, 86.5, 81.8, 75.5, 75.5, 70.4, 70.0, 69.8, 67.9, 67.8, 64.9, 59.0, 58.9, 36.1, 16.7; LCMS (ESI⁺): 604.1 [M + H]⁺.

N-(2-Methoxyethoxycarbonyl)-MTA (**2f**, LH1209): Yield, 70 mg (62%) obtained from 170 mg (0.28 mmol) of **3f**. ^1H NMR (400 MHz, Chloroform-*d*) δ (ppm): 8.52 (s, 1H), 8.12 (s, 1H), 5.94 (d, $J = 5.0$ Hz, 1H), 4.69 (t, $J = 5.2$ Hz, 1H), 4.38 – 4.34 (m, 1H), 4.33 – 4.30 (m, 3H), 3.58 (dd, $J = 8.8, 4.2$ Hz, 4H), 3.31 (s, 3H), 2.77 (dq, $J = 13.9, 6.8, 5.6$ Hz, 2H), 2.06 (s, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ (ppm): 152.4, 150.9, 150.7, 149.4, 141.7, 122.3, 89.9, 84.4, 74.7, 73.3, 70.4, 64.9, 58.9, 36.8, 16.6; LCMS (ESI⁺): 400.1 [M + H]⁺.

Stability Assay of MTA prodrugs in Phosphate Buffer

The stock solution of the prodrug (10 mM in DMSO) was diluted 10-fold to give a 1 mM working solution in 10% DMSO. 100 μL of the 1 mM solution was added to 875 μL of 50 mM phosphate buffer containing 1 mM EDTA, pH 7.4 and incubated at 37 °C for 5 minutes in an Eppendorf vial. The vial was then centrifuged for 5 minutes and the supernatant was split into 3 fractions of 292.5 μL , one for stability in phosphate buffer, one for activation in human liver microsomes and one for activation in mouse liver microsomes.

The fraction for analyzing stability was mixed with 7.5 μL of deionized water and incubated at 37 °C for 3 days. Time points were collected after every 0 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 3 h, 5 h, 8 h, 10 h, 12 h, 24 h, 48 h, 72 h. At each time point, 20 μL aliquots were taken and diluted with 40 μL cold acetonitrile and centrifuged for 5 mins. 40 μL of the supernatant were taken and diluted with 40 μL deionized water and frozen before injecting on the HPLC. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50 x 4.6 mm) column and a gradient of 10 - 90% acetonitrile containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with detection wavelength of 220 and 254 nm.

In vitro Activation Assay in Human and Mouse Liver Microsomes [18]

The mouse and human liver microsomes were obtained from Xeno Tech with a protein concentration of 20 mg/ml and stored in Eppendorf vials at -70 °C. Stock solution of 1 mM compound in 1 mL DMSO was diluted 10-fold to give a 1 mM solution containing 10% DMSO. 100 μL of the 1 mM solution was added to 875 μL of 50 mM phosphate buffer containing 1 mM EDTA, pH 7.4 and incubated at 37 °C for 5 minutes in an Eppendorf vial. The vial was then centrifuged for 5 minutes and the supernatant was split into 3 fractions of 292.5 μL , one for stability in phosphate buffer, one for activation in human liver microsomes and one for activation in mouse liver microsomes. The fraction for analyzing activation was mixed with 7.5 μL of either the human or mouse liver microsomes and incubated at 37 °C for 2 days.

Time points were collected after every 0 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 3 h, 5 h, 8 h, 10 h, 12 h, 24 h, and 48 h. At each time point, 20 μ L aliquots were taken and quenched with 40 μ L cold acetonitrile and stored on ice for 30 mins before centrifugation for 5 mins. 40 μ L of the supernatant were taken and diluted with 40 μ L deionized water and frozen before injecting on the HPLC. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50 x 4.6 mm) column and a gradient of 10 - 90% acetonitrile containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with a detection wavelength of 220 and 254 nm. The half-life was calculated from the first order rate equation of the conversion of prodrug to MTA.

Oxidative Activation Assay in presence of NADPH

Stock solution (10 mM in DMSO) was diluted 10-fold to give a solution of 1 mM in 10% DMSO. 100 μ L of the 1 mM solution was added to 875 μ L of 50 mM phosphate buffer containing 1 mM EDTA, pH 7.4 and incubated at 37 °C for 5 minutes in an Eppendorf vial. The vial was then centrifuged for 5 minutes and the supernatant was split into 2 fractions, each of 292.5 μ L, one for oxidative activation in mouse liver microsomes and one for human liver microsomes. Each of the fractions was mixed with 20 μ L of NADPH and either 20 μ L of mouse liver microsomes or human liver microsomes and incubated at 37 °C for 4 h. At the end of incubation, 20 μ L aliquots were taken and quenched with 40 μ L cold acetonitrile and stored on ice for 30 mins before centrifugation for 5 mins. 40 μ L of the supernatant were taken and diluted with 40 μ L deionized water and frozen prior to HPLC analysis. The samples were analyzed using a Perkin Elmer Series 2000 HPLC with a Phenomenex Kinetix C18 (50 x 4.6 mm) column and a gradient of 10 - 90% acetonitrile containing 0.1% TFA in 10 min at a flowrate of 0.6 mL/min was used with a detection wavelength of 220 and 254 nm. The half-life was calculated from the first order rate equation of the conversion of prodrug to MTA.

Abbreviations Used

AMP, adenosine monophosphate; APRT, adenine phosphoribosyltransferase; BNPP, bis(4-nitrophenyl)-phosphate; 5-FU, 5-fluorouracil; HLM, human liver microsome; HPLC, high performance liquid chromatography; IC_{50} , concentration required to produce 50% inhibition; LC-MS, liquid chromatography-mass spectrometry; MLM, mouse liver microsome; MTA, 5'-methylthioadenosine; MTAP, MTA phosphorylase; NADPH, nicotinamide adenine dinucleotide phosphate; PRPP, phosphoribosyl-1-pyrophosphate; 6-TG, 6-thioguanine; TLC, thin layer chromatography.

Declarations

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Declaration of Conflicting Interests

The authors declare no competing interests.

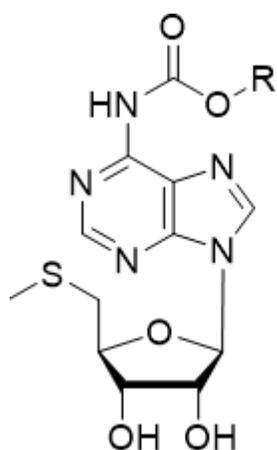
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Tables

Table 2. Stability and activation of *N*-(alkyloxy)carbonyl-MTA prodrugs **2a-f**



Compound ID	R	$t_{1/2}$ (h) ^a		
		Buffer	MLM	HLM
2a (LH1201)	CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	>72 (< 10 %)	1.2	>48 (~44 %)
2b (LH1205)	CH ₂ CH ₂ CH ₂ CH ₃	>72 (< 10 %)	9.4	>48 (~19 %)
2c (LH1206)	CH(CH ₃) ₂	>72 (< 10 %)	>48 (< 10 %)	>48 (< 10 %)
2d (LH1207)	CH ₂ CH ₃	>72 (< 10 %)	>48 (< 10 %)	>48 (< 10 %)
2e (LH1208)	CH ₂ CH ₂ CH ₃	>72 (< 10 %)	40	>48 (< 10 %)
2f (LH1209)	CH ₂ CH ₂ OCH ₃	>72 (< 10 %)	>48 (~15%)	>48 (< 10 %)

^aThe half lives of prodrugs were measured using HPLC by following their disappearance at 100 mM and 37 °C in 50 mM Phosphate buffer containing 1 mM EDTA, pH=7.4, in the absence (buffer) or presence of 0.5 mg/ml mouse liver microsome (MLM) or human liver microsome (HLM). All compounds were followed for 72 h in phosphate buffer and 48 h in the presence of MLM or HLM with % change indicated in brackets at the end of incubation when <50% change were observed.