Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1673–1678

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

N^3-METHYL-MAFOSFAMIDE AS A CHEMICALLY STABLE, ALTERNATIVE PRODRUG OF MAFOSFAMIDE

Ki-Young Moon* and Chul-Hoon Kwon

Department of Pharmaceutical Sciences, College of Pharmacy, St. John's University, Jamaica, New York, 11439, U. S. A.

Received 3 March 1998; accepted 26 May 1998

Abstract: The presence of an alkyl substituent at N^3 in the oxazaphosphorine ring stabilizes N-substituted 4-(alkylthio)cyclophosphamides from spontaneous decomposition. Based on this finding, N^3-methyl-mafosfamide was synthesized and examined as a chemically stable, biooxidative prodrug of mafosfamide. This prodrug was stable in aqueous buffer (pH 7.4, 37 °C) and underwent N-demethylation in a time dependent manner when incubated with rat hepatic microsomes. N^3-Methyl-mafosfamide was 10-fold more cytotoxic in vitro than cyclophosphamide against mouse embryo Balb/c 3T3 cells (LC_{50} = 3.6 μM). Preliminary in vivo antitumor evaluation against L1210 leukemia in mice showed that this prodrug was active [Increase of life span (ILS) > 29 %].

Cyclophosphamide (CP, NSC-26271), a widely used anticancer agent, is a prodrug that is activated by hepatic microsomal mixed-function oxidase (MFO) catalyzed C_4-hydroxylation. The major metabolic activation pathway of CP is shown in Scheme 1. Although CP has shown a relatively high oncotoxic selectivity and therapeutic efficacy,[1-7] a number of side effects are accompanied largely due to the liberated acrolein, a byproduct of the β-elimination. Among the side effects, the most serious problem is the urotoxic effect (i.e., hemorrhagic cystitis that is open dose-limiting in CP treatment).[8,9]

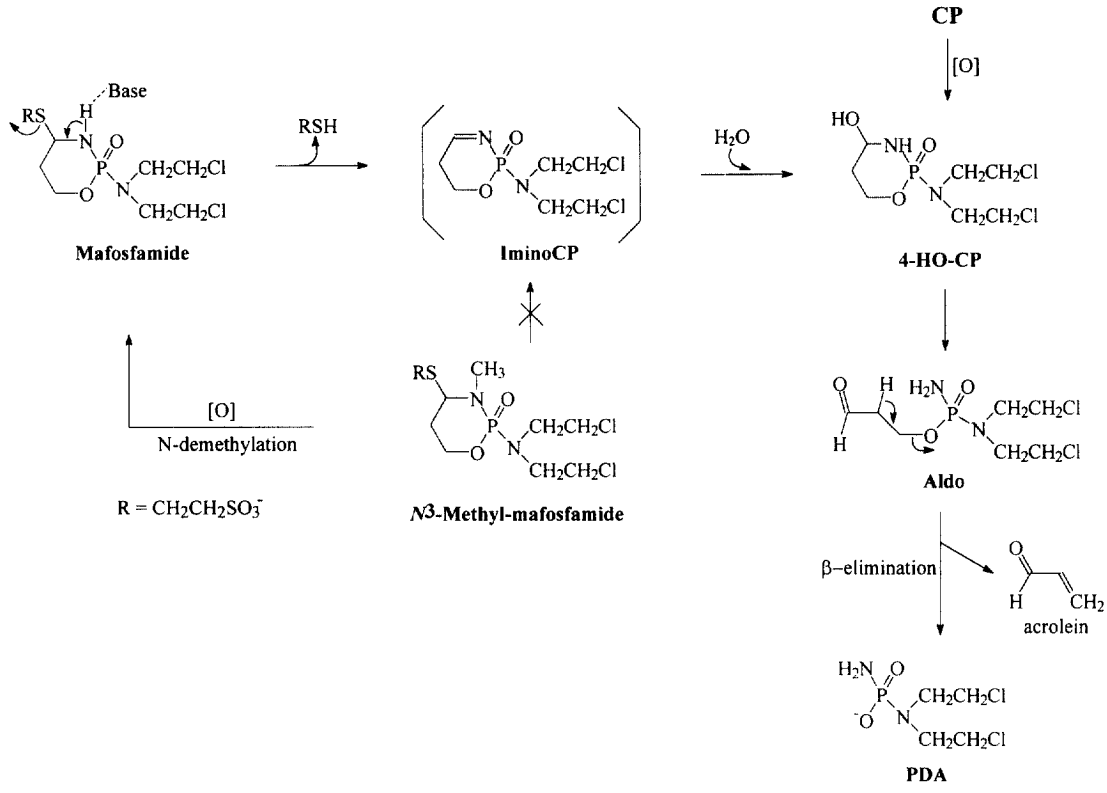
We previously reported[10] that the *N*-methyl-4-(alkylthio)cyclophosphamide derivatives, N^3-methyl-4-(*n*-propylthio)-CP and N^3-methyl-4-(diethyldithiocarbamoyl)-CP, were chemically stable until oxidatively N-demethylated in the presence of hepatic microsomal P-450 enzymes and served as thiol-containing chemically stable biooxidative prodrugs of 4-HO-CP (Scheme 1).

Many studies have shown that various sulfhydryl-containing compounds have protective roles in acrolein-induced toxicities without interfering in CP anticancer activity.[11] Among these thiol containing compounds, MESNA (2-mercaptoethanesulfonate)[12] is relatively nontoxic and has shown to provide protective roles against the acrolein-induced side effects of CP. Mafosfamide (ASTA Z7557), originally introduced as a chemically stable derivative of 4-HO-CP, contains a thiol, viz., MESNA (2-mercaptoethanesulfonate) at C_4 on the oxazaphosphorine ring. Its stability in aqueous buffer is highly dependent on pH.[13]

*E-mail: kmoon@mail.ncifcrf.gov

0960-894X/98/$19.00
PII: S0960-894X(98)00287-X

Scheme 1



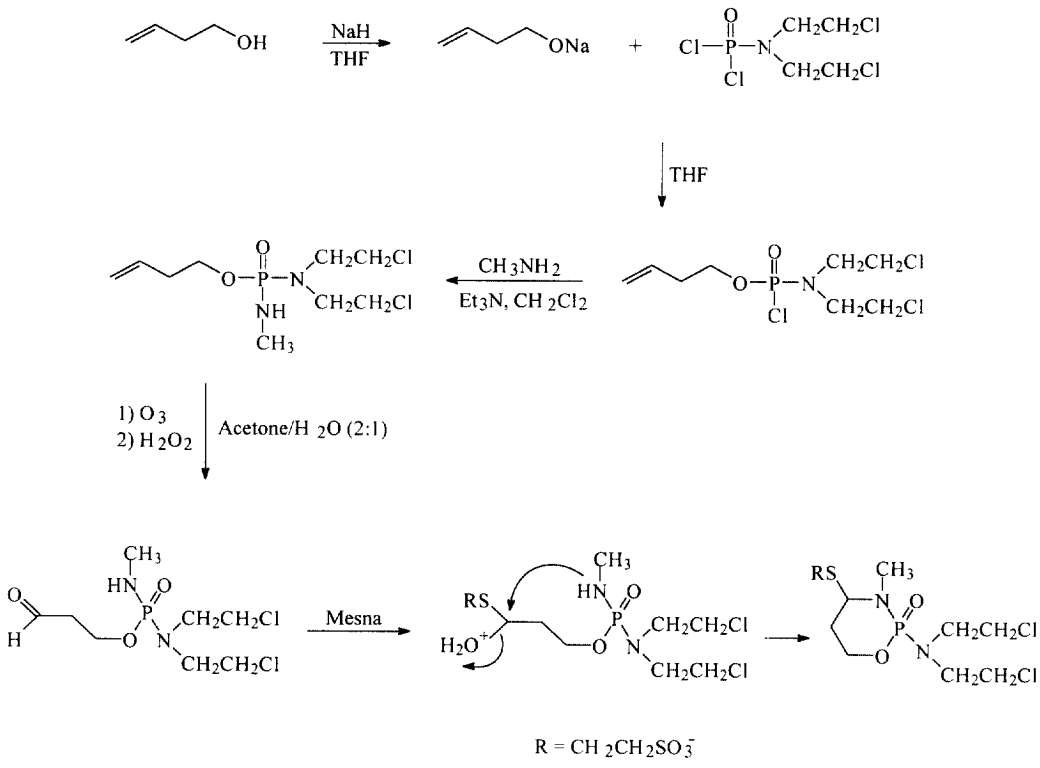
The mechanistic details of its activation have been previously discussed.[14] Briefly, mafosfamide undergoes a rapid decomposition in aqueous buffer or plasma at pH 7.4 and 37 °C. The kinetics of the reaction are complex: (1) base-catalyzed expulsion of the thiol (MESNA) to a transient iminocyclophosphamide (iminoCP) intermediate, and (2) rapid reaction of the intermediate with water to form 4-HO-CP (Scheme 1). Preclinical evaluation of toxicity and therapeutic efficacy[7, 15] indicate that the antitumor activity of mafosfamide is similar to that of CP, but mafosfamide is also marginally effective against the CP resistant P388 cell line[16] and shows less myelo- and urotoxicity than CP.[17] However, phase I clinical studies have shown that mafosfamide causes a severe local toxicity along the injected vein and acute irritation of mucose membranes.[18] This is presumably due to the high concentration of the 4-HO-CP and its subsequent toxic metabolites. Therefore, the premature catabolism of 4-(alkylthio)cyclophosphamide derivatives severely limits their potential use in cancer chemotherapy, despite reduced nephrotoxicity when compared with CP. These observations led us to develop N^3-methyl-4-MESNA-substituted cyclophosphamide, one such compound, N^3-methyl-mafosfamide was synthesized and examined as a chemically stable, biooxidative prodrug of mafosfamide. The thiol liberated from this compound is expected to trap the byproduct, acrolein, and improve therapeutic efficacy. This prodrug of mafosfamide should be pharmacologically inert itself and must undergo N-demethylation in vivo before it

can generate mafosfamide and its subsequent cytotoxic metabolites. The synthesis and mechanistic studies on the activation of N^3-methyl-mafosfamide and its preliminary evaluation of usefulness as an alternative prodrug of mafosfamide are described herein.

Chemistry

N^3-Methyl-4-(sulfonylethylthio)cyclophosphamide (N^3-methyl-mafosfamide) was synthesized by directly adding the MESNA to the ozonide reaction mixture of *O*-(3-butenyl)*N,N*-bis(2-chloroethyl)-*N'*-methylphosphorodiamidate[19] (Scheme 2).

Scheme 2



The target product was obtained as a fluffy, hygroscopic powder in 7% yield.[20] The pH of the reaction mixture gradually changed from 7.9 to 4.8 as the reaction proceeded. It was therefore not necessary to add trifluoroacetic acid to facilitate the formation of the cyclized final product.[10] The decrease in pH was attributed to the formation of acidic N-CH_3-PDA from the decomposition of N-CH_3-Aldo. The target compound was purified by flash column chromatography using a gradient elution solvent system (CH_2Cl_2:MeOH, 9:1 → CH_2Cl_2:MeOH, 1:1) and followed by preparative TLC (CH_2Cl_2:MeOH, 9:1). The ^{1}HNMR revealed a large H_4-P coupling constant ($J_{C4\text{-}H,\,P}$ = 22 Hz). Therefore, the product was assigned as the *cis*-isomer.[10]

Results and Discussion

N^3-Methyl-mafosfamide was examined as a chemically stable, alternative biooxidative prodrug of mafosfamide. N^3-Methyl-mafosfamide was stable in aqueous buffer under physiological conditions (pH 7.4 and 37 °C) for at least 24 h and did not show significant alkylating activity as assayed by reaction with NBP [4-(4-nitrobenzyl)pyridine].[21] However, when this compound was incubated in the presence of rat liver microsomes and NADPH-generating system, a time-dependent generation of alkylating species was observed (0.32 ± 0.07 equiv of mechlorethamine/g of wet weight liver/h) (CP, 0.16 ± 0.02 equiv of mechlorethamine/g of wet weight liver/h).[21] The extent of N-demethylation was determined by following the rate of formaldehyde production from microsomal incubation of this compound.[21] The rate of formaldehyde formation was linear with incubation time (0.86 ± 0.10 equiv of formaldehyde/g of wet weight liver/h). It was concluded, based on the time dependent formation of an alkylating species and formaldehyde, that N^3-methyl-mafosfamide underwent the proposed bioactivation to mafosfamide, which subsequently generates the thiol and 4-HO-CP (Scheme 1).

In vitro cytotoxicity of N^3-methyl-mafosfamide was initially evaluated against mouse embryo Balb/c 3T3 cells and compared with those of CP (LC_{50} = 37.6 μM) and PDA (LC_{50} = 3.0 μM).[21] 3T3 cells, known to possess MFO activity,[22] are therefore ideal for compounds that require MFO-mediated activation such as in this case. N^3-Methyl-mafosfamide showed a ten fold greater cytotoxicity than CP.

Subsequently, preliminary in vivo antitumor activity of N^3-methyl-mafosfamide was evaluated against L1210 leukemia in mice. N^3-Methyl-mafosfamide showed a marginal activity (ILS = 29 %) at 1.08 mmol/kg (single dose) despite the high cytotoxicity against Balb/c 3T3 cells (LC_{50} = 3.6 μM). A possible explanation for this could be the low lipophilicity of this compound resulting in insufficient intracellular uptake and/or extracellular elimination of N^3-methyl-mafosfamide.

In summary, N^3-methyl-mafosfamide was prepared to evaluate possible oxidative N-demethylation to yield mafosfamide, 4-HO-CP and MESNA. N^3-Methyl-mafosfamide was chemically stable, but was found to undergo oxidative N-demethylation, presumably by hepatic microsomal MFO systems. Cytotoxicity profiles of N^3-methyl-mafosfamide in vitro proved to be better than those of CP. Preliminary antitumor screening against L1210 leukemia in mice suggested that N^3-methyl-mafosfamide showed a marginal in vivo activity. Further studies exploring the clinical usefulness of N^3-methyl-mafosfamide are required to evaluate whether this prodrug can be an useful anticancer compound with uroprotective effect.

Acknowledgement. We thank Dr. F. N. Shirota, Department of Veterans Affairs Medical Center, Minneapolis, Minnesota for in vivo studies.

References

1. Friedman, O. M.; Myles, A.; Colvin, M. *Adv. Cancer Chemother.* **1979**, 1, 143.
2. Brock, N.; Hohorst, H.-J. Z. *Krebsforsch. Klin. Onkol.* **1977**, 88, 185.

3. Brock, N. *Cancer Treat. Rep.* **1976**, 60, 301.
4. Sladek, N. E. *Pharmacol. Ther.* **1988**, 37, 301.
5. Jardine, I.; Fenselau, C.; Appler, M.; Kan, M.-N.; Brundrett, R. B.; Colvin, M. *Cancer Res.* **1978**, 38, 408.
6. Struck, R. F.; Kirk, M. C.; Witt, M. H.; Laster, W. R. Jr. *Biomed. Mass Spectrom.* **1975**, 2, 46.
7. Alberts, D.S.; Einspahr, J. G.; Struck, R. F.; Bignami, G.; Young, L.; Surwit, E. A.; Salmom, S.E. *Invest. New Drugs* **1984**, 2, 141.
8. Brock, N.; Stekar, J.; Pohl, J.; Niemeyer, U.; Scheffler, G. *Arzneim.-Forsch.* **1979**, 29, 659.
9. Cox, P. J. *Biochem. Pharmacol.* **1979**, 28, 2045.
10. Moon, K-. Y.; Shirota, F. N.; Baturay, N.; Kwon, C-. H. *J. Med. Chem.* **1995**, 38, 848.
11. Berrigan, M. J.; Marinello, A. J.; Pavelic, Z.; Williams, C. J.; Struck, R. F.; Gurtoo, H. C. *Cancer Res.* **1982**, 42, 3688.
12. (a) Brock, N.; Pohl, J.; Stekar, J.; Scheef, W. *Eur. J. Cancer Clin. Oncol.* **1982**, 18, 1377. (b) Schmahl, D.; Habs, M. R. *Cancer Treat. Rev.* **1983**, 10, Suppl. A, 57. (c) Millar, B. C.; Millar, J. L.; Clutterbuck, R.; Jinks, S. *Cancer Treat. Rev.* **1983**, 10, Suppl. A, 63. (d) Wagner, T.; Zink, M.; Schwieder, G. J. *Cancer Res. Clin. Oncol.* **1987**, 113, 160. (e) Bernacki, R. J.; Bansal, S. K.; Gurtoo, H. L. *Cancer Res.* **1987**, 47, 799.
13. Niemeyer, U.; Engel, J.; Scheffler, G.; Molge, K.; Sauerbier, D.; Weigert, W. *Invest. New Drugs* **1984**, 2, 133.
14. Kwon, C.-H.; Borch, R. F.; Engel, J.; Niemeyer, U. *J. Med. Chem.* **1987**, 30, 395.
15. (a) Klein, H. O.; Wickramanayake, P. D.; Christian, E.; Coerper, C. *Invest. New Drugs* **1984**, 2, 191. (b) Berger, M. R.; Bedford, P.; Zeller, W. J.; Kaufmann, M. *Invest. New Drugs* **1984**, 2, 181. (c) Nowrousian, M. R.; Schmidt, C. G. *Invest. New Drugs* **1984**, 2, 207.
16. Atassi, G.; Hilgard, P.; Pohl, J. *Invest. New Drugs* **1984**, 2, 169.
17. Pohl, J.; Hilgard, P.; Jahn, W.; Zechel, H. J. *Invest. New Drugs* **1984**, 2, 201.
18. (a) Bruntsch, U.; Groos, G.; Hiller, T. A.; Wandt, H.; Tigges, F.-J.; Gallmeier, W. M. *Invest. New Drugs* **1985**, 3, 293. (b) Abele, R.; Aapro, M. S.; Haefliger, J-M.; Alberto, P. *Cancer Chemo. Pharmaco.* **1986**, 16, 182.
19. Kwon, C.-H.; Borch, R. F. *J. Med. Chem.* **1989**, 32, 1491.
20. N^3-Methyl-*cis*-4-(sulfonylethylthio)cyclophosphamide, 2-mercaptoethanesulfonic acid, cyclohexylamine salt (N^3-methyl-*cis*-mafosfamide). 2-Mercaptoethanesulfonic acid, sodium salt (8.25 g, 0.05 mol) was dissolved in 40 mL of water (pH 4.8) and further acidified with concentrated HCl to pH 0.2-0.3. The mixture was evaporated to give clear crystals. The crystals were dissolved in absolute ethanol (100 mL) and the salts were removed by filtration. Cyclohexylamine (5.95 g, 6.86 mL, 0.06 mol) was added dropwise and the mixture was stirred for 10 min at room temperature. The reaction mixture was evaporated to give a thin-

purple oil. The oil was crystallized from anhydrous diethyl ether to give 2-mercaptoethanesulfonic acid, cyclohexylamine salt as a slightly purple color solid (10.5 g, 87 % yield) which was used directly for the synthesis of the target compound. *O*-(3-Butenyl)*N,N*-bis(2-chloroethyl)-*N'*-methylphosphorodiamidate (2.59 g, 0.01 mol) was dissolved in acetone/water (150 mL, 2:1) and ozonized at ice bath temperature for 15 min. 2-Mercaptoethanesulfonic acid, cyclohexylamine salt (9.66 g, 0.04 mol) prepared above was dissolved in acetone:water (45 mL, 2:1) and added to the reaction solution. The reaction was allowed to proceed at ice bath temperature for 3.5 h. As the reaction proceeded, the pH of the mixture was gradually changed from 7.9 to 4.8. The reaction mixture was directly extracted with EtoAc (9 x 50 mL). The organic extracts were pooled, dried over Na_2SO_4, and filtered. The filtrate was evaporated in vacuo to give a thin yellow semi-solid (5.92 g). The semi-solid was crystallized from anhydrous diethyl ether/acetone/ethanol to give a slightly yellow solid. This was identified as unreacted Mesna, cyclohexylamine salt and therefore discarded by filtration. The filtrate was evaporated to give a yellow fluffy, hygroscopic powder. The hygroscopic powder was further crystallized from ethanol/anhydrous diethyl ether/acetone to remove unreacted Mesna, cyclohexylamine salt. The filtrate was concentrated to a dark yellow oil which was directly applied to a silica gel column (40 μm, 3 x 15 cm) packed dry, wetted , and eluted with CH_2Cl_2:MeOH (9:1) under a positive pressure (15 psi). A total of 37 x 10 mL fractions were collected. The eluting solvent mixture was then changed to CH_2Cl_2/MeOH (1:1) and a total of 27 x 10 mL fractions were collected. The fractions, containing the desired compound (#5-26), were pooled and evaporated to give 0.84 g of a slight yellow semi-solid. The semi-solid was further purified by a preparative TLC: the semi-solid was dissolved in an equal volume of methanol and eluted with CH_2Cl_2/MeOH (9:1). The band containing the desired product was scraped and extracted with absolute ethanol (150 mL) to obtain 0.39 g of a thin yellow oil. The oil was diluted with acetone/anhydrous diethyl ether resulting in a milky solution which was evaporated to give 0.37 g of a fluffy, hygroscopic powder: Yield, 7.2 %; TLC Rf= 0.31 in CH_2Cl_2:MeOH (9:1); ^{1}H NMR ($CDCl_3$) δ 4.72 - 4.88 (1H, m, H_{6a}), 4.42 (1H, ddd, $J_{C4\text{-}H,\ P}$ = 22.0 Hz, H_4), 4.15 - 4.30 (1H, m, H_{6e}), 3.65 (4H, m, CH_2Cl), 3.40 (4H, m, CH_2N), 3.14 (4H, m, CH_2CH_2S), 2.98 (3H, br, $+NH_3$), 2.75 (3H, d, $J_{H,\ P}$ = 10.1 Hz, NCH_3), 2.35-2.52 and 1.95-2.25 (2H, m, H_5), 1.80 (2H, m, cyclohexyl H), 1.65 (2H, m, cyclohexyl H), 1.05-1.55 (7H, m, cyclohexyl H). Anal. ($C_{16}H_{34}Cl_2N_3O_5PS_2$. 0.05 mesna. 0.5 H_2O): Cal'd, C: 36.78, H: 6.76, N: 7.97; Found, C: 36.76, H: 6.62, N: 7.23.

21. Kwon, C.-H.; Moon, K.-Y.; Baturay, N.; Shirota, F. N. *J. Med. Chem.* **1991**, 34, 588.

22. Baturay, N. Z.; Trombetta, L. D. *Arch. Dermatol. Res.* **1988**, 280, 443.