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When alcohol is the answer: trapping, identifying and quantifying simple alkylating species in aqueous environments

P. G. Penketh^{1,3}, K. Shyam², R. P. Baumann², Rui Zhu², K. Ishiguro¹, A. C. Sartorelli², and E. S. Ratner¹

¹Department of Obstetrics, Gynecology & Reproductive Sciences, Yale University School of Medicine, New Haven, CT. 06520-8063 USA

²Department of Pharmacology, Yale University School of Medicine, New Haven, CT. 06520-8066 USA

Abstract

Alkylating agents are a significant class of environmental carcinogens as well as commonly used anticancer therapeutics. Traditional alkylating activity assays have utilized the colorimetric reagent 4-(4-nitrobenzyl)pyridine (4NBP). However, 4NBP based assays have a relatively low sensitivity towards harder, more oxophilic alkylating species and are not well suited for the identification of the trapped alkyl moiety due to adduct instability. Herein we describe a method using water as the trapping agent which permits the trapping of simple alkylating electrophiles with a comparatively wide range of softness/hardness and permits the identification of donated simple alkyl moieties.

Introduction

All alkylating agents from therapeutic agents to environmental toxins are electrophilic species, or precursors thereof, that transfer alkyl moieties to DNA (and other biological nucleophiles) to generate anticancer and/or toxic effects (1-3) (Fig 1 panel A). Anticancer activity generally arises by exploiting specific defects in DNA repair (or differences in DNA damage tolerance) between tumor cells and DNA repair competent normal cells (1-3). A common misconception in biological sciences is that all alkylating agents will react avidly with ‘strongly nucleophilic’ groups (especially thiols), however this is not the case (4,5). The concept of ‘hard’ and ‘soft’ nucleophiles and electrophiles was introduced to explain relative electrophile/nucleophile reaction preference (4). Hard electrophiles possess a high positive charge density, and tend to react via S_N1 reaction mechanisms with hard nucleophiles (those with high negative charge density) such as oxygen centers. Soft electrophiles have a low charge positive density or are easily polarized and tend to react via S_N2 reaction mechanisms with soft nucleophiles (those with a low negative charge density or are easily polarized) such as thiols (4). Reaction activation energy is the lowest between

³To whom all correspondence should be addressed. Phone: (203)785-4524; philip.penketh@yale.edu or philip.penketh@gmail.com.

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electrophile/nucleophile pairs with closely matching degrees of hardness/softness (4). This behavior imparts the preference of electrophilic alkylating species for different nucleophilic sites within DNA (4). The reason abundant DNA alkylations are sometimes seen at very diverse sites of hardness/softness with anticancer agents like the chloroethylnitrosoureas (CNUs) is because they frequently produce a range of alkylating electrophiles with very different degrees of hardness/softness due to the occurrence of multiple decomposition routes (6,7). The softer species generated by the CNUs contribute little towards their therapeutic activity, and react predominantly at the *N*-7 position of guanine (the softest nucleophilic site within DNA), whilst the harder, therapeutically more important CNU derived electrophiles react more efficiently at the *O*-6 position of guanine (the hardest nucleophilic base localized site within DNA)(4). The commonly used colorimetric alkylating activity reagent 4-(4-nitrobenzyl)pyridine (4NBP) (Fig 1 panel A) (8-10) features a soft pyridine nitrogen as the nucleophilic target and in that regard it resembles the *N*-7 position of DNA guanine. Thus, 4NBP will give a strong signal with electrophiles that primarily target guanine *N*-7 but will have a much lower sensitivity towards harder oxophilic electrophiles that target guanine *O*-6. In the case of BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea, carmustine) the majority of the alkylation signal recorded using 4NBP likely represents reaction with electrophiles not responsible for a significant proportion of the agent's therapeutic (or mutagenic) activity (6,7). This weakness will preclude good correlations between the alkylating activity determined using 4NBP and biological activity with some classes of agents.

Method Development and Rationale

The amount of alkylating activity trapped by a nucleophile depends on two main factors: the preference of the electrophile for the trapping nucleophile; and the concentration of the trapping nucleophile (higher concentrations giving more efficient trapping) (4). In biological studies involving the activation/metabolism of targeted alkylating agent prodrugs by enzymes or cells, the presence of very high concentrations of any foreign agent/electrophile-trap could influence the system and compromise the experiment. The conscription of a suitable, ubiquitously occurring endogenous molecule as an electrophile trap would prevent this issue. Water is generally overlooked as a potential trap despite its ~ 55 M concentration. This extreme concentration effectively broadens its functionality as an electrophile trap. Thus relatively soft thiophilic alkylating electrophiles can be trapped as their respective alcohols (Fig 1, panel A) by water despite water's innate preference for harder electrophiles, e.g., dimethylsulfate alkylates water to produce methanol with a $t_{1/2}$ of 7.5 min (37°C, pH 7.4) (11). We have been particularly interested in hard chloroethylating, ethylating, vinylating, hydroxyethylating and methylating electrophiles produced by various anticancer nitrosoureas and sulfonylhydrazines (3). These electrophiles will alkylate water to yield 2-chloroethanol, ethanol, acetaldehyde, ethylene glycol and methanol, respectively. All these products are alcohols with the exception of acetaldehyde, (produced by the tautomerization of vinyl alcohol). These small, freely miscible, and relatively lipophilic molecules can easily pass through cell membranes and equilibrate with the bulk phase when generated within cells under tissue culture conditions. Simple alcohols lack any strong spectroscopic features and are relatively unreactive, therefore it is necessary to transform them into more

chromophoric derivatives for easy detection. *Pichia pastoris* alcohol oxidase is a relatively non-specific alcohol oxidase which efficiently oxidizes short-chain, linear aliphatic alcohols to their respective aldehydes using ambient dissolved O₂ (12). While methanol and ethanol are the preferred substrates other alcohols are still oxidized by this enzyme at slower, but still very acceptable, rates (12). Thus we decided to utilize this enzymatic oxidation to transform the resultant alcohols in to their respective aldehydes which could then be identified and quantified after conversion to their corresponding 2,4-dinitrophenylhydrazones by reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH, Brady's reagent) (13). The 2,4-dinitrophenylhydrazone products are highly chromophoric and their extinction coefficient at 370 nm is essentially independent of the aldehyde derived moiety; additionally they are easily resolved by HPLC (Table 1). Thus the identity of the donated alkylating moiety (which can vary in some cases due to multiple reaction pathways (6,7)) can be determined by comparison of the hydrazone's elution time with that of hydrazone standards synthesized from authentic alcohol or aldehyde samples. The identification of vinylating species does not require the use of alcohol oxidase since vinyl alcohol spontaneously tautomerizes to acetaldehyde. A mixture of vinylating and ethylating activities can be individually quantified by determining the ethanal-2,4-dinitrophenylhydrazone hydrazone yields without an alcohol oxidase step to ascertain vinylating activity and then repeating the experiment with an alcohol oxidase step to acquire a value for combined ethylating and vinylating activities (ethylating only activity determined by difference).

Potential complications

We have encountered two readily surmountable problems using this methodology to date. The first concerns over oxidation, this problem is primarily faced in the determination of methylating species using *Pichia pastoris* alcohol oxidase. Methanol is a very good substrate for *Pichia pastoris* alcohol oxidase, unfortunately the initial oxidation product formaldehyde is also a moderate substrate being further oxidized to formic acid (14). Therefore, in prolonged incubations the signal generated due to formaldehyde eventually decreases with time. It is therefore prudent in the determination of methylating activity to either use an alcohol oxidase with either a lower affinity for formaldehyde such as from *Poria contigua* (15) and/or avoid excessive oxidation by the use of relatively short low temperature incubations prior to hydrazone formation (Table 1). Under the detailed conditions the alcohols, with the exception of ethylene glycol, are nearly stoichiometrically converted to their respective aldehydes without significant over oxidation. Ethylene glycol is a particularly poor substrate for *Pichia pastoris* alcohol oxidase and was transformed to glyoxal with a ~ 50% yield under the specified conditions. The second problem is restricted to reaction mixtures that contain high concentrations of thiols. If thiols are abundant in the reaction mixture they may react with the aldehydes generated by the alcohol oxidase to form hemithioacetals or thioacetals (16) preventing the aldehyde from reacting readily with 2,4-DNPH. This problem is easily circumvented by adding a molar excess (with respect to thiol) of N-ethylmaleimide (NEM) prior to the alcohol oxidase oxidation step to titrate the thiols (NEM does not significantly inhibit alcohol oxidase).

Assay conditions

Experimental mixtures to be assayed were diluted with 20 mM Tris-HCl, 1.0 mM EDTA buffer pH 7.4 to give estimated alcohol concentrations in a range between 5-100 μ M. One percent by volume of a 250 mM NEM solution in DMSO was then added (if required, due to the presence of thiols) and the mixtures incubated for 5 minutes at 40°C. Alcohol oxidase was then added to these mixtures and they were incubated, with regular stirring to maintain oxygenation, under various conditions depending upon the type of alcohol being determined (Table 1). Alcohols which were poorer alcohol oxidase substrates required longer incubation at higher temperatures with greater enzyme levels for near stoichiometric oxidation to their respective aldehydes.

To generate the respective hydrazones 0.5 ml of the oxidized alcohol solutions were mixed with an equal volume of 0.04% 2,4-DNPH in acetonitrile and 50 μ L of 1 M HClO₄ was then added to catalyze hydrazone formation. These mixtures were then incubated at 40°C for 5 minutes, centrifuged at 10,000 g for 5 minutes to remove any precipitant, and the supernatants analyzed promptly by HPLC. Two HPLC conditions were used. The first HPLC protocol utilized a 250 mm $\times$ 4.6 mm Varian Microsorb 100-5 C-18 reverse phase column (Varian Inc, Lake Forest, California, USA), a constant composition elution buffer composed of 30 mM potassium phosphate (pH 5.4)/acetonitrile (52.5% by volume), and a flow rate of 0.8 mL/min, with the elutant monitored at 370 nm. With this protocol unreacted 2,4-DNPH, and the hydrazones corresponding to formaldehyde, acetaldehyde, and 2-chloroacetaldehyde eluted at 6.5, 11.1, 14.9, and 16.3 min respectively. The second HPLC protocol was used to quantify the hydrazone corresponding to glyoxal and differed only in the acetonitrile content which was increased to 75% (to speed elution) resulting in an elution time of 9.1 min. Assayed alcohol (and aldehyde) standards gave a linear relationship between the area under the corresponding elution peak (at 370 nm) versus concentration in the tested range (5-100 μ M) Fig 1, panel B. The limit of detection using a UV/visible detector (without any sample concentration) is $\sim$ 5 μ M, however 2,4-DNPH hydrazones are very electroactive and the use of an amperometric HPLC detector should increase the sensitivity by $\sim$ 20-fold (17).

Using this protocol we examined the effects of 1-thioglycerol and inorganic phosphate both in the 0-20 mM range on the chloroethanol yields from 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE). 90CE is the short-lived ($T_{1/2} \sim$ 30 s) species generated by the investigational anticancer prodrugs laromustine (cloretazine, onrigin, VNP40101M, 101M, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine), KS119 (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine), and RKS119W (VNP40541, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(3-phospho-4-nitrophenyl)ethoxy]carbonyl]hydrazine) that leads via the *O*-6 chloroethylation of DNA guanine to DNA interstrand cross-links (3) (Fig 1, panel C). 10 μ L of a 100 mM solution of 90CE in DMSO was added to 1 mL of 20 mM Tris-HCl, 1.0 mM EDTA buffer pH 7.4 containing various concentrations (0 - 20 mM) of either 1-thioglycerol or inorganic phosphate. This mixture was incubated at 37°C for 10 min to allow the 90CE to fully react. This mixture was then diluted 10-fold with 20 mM Tris-HCl, 1.0 mM EDTA buffer pH 7.4 and assayed for chloroethanol as described above (Fig 1, panel C). It can be seen that high

concentrations of 1-thioglycerol have very little effects on the 2-chloroethanol yields from 90CE. This is the expected result as the hard oxophilic chloroethylating species generated should have little affinity for thiols, and this observation correlates with the very minimal thiol inhibition of DNA cross-linking by 90CE previously reported (5,18). The very low affinity of 90CE derived hard chloroethylating electrophiles for thiols also highlights the weakness of using soft electrophilic traps, especially at relatively low concentrations, to quantify harder alkylating species. In contrast, the presence of inorganic phosphate strongly decreased the yields of chloroethanol from 90CE and this correlates with a strong phosphate inhibition of 90CE dependent DNA cross-linking reported earlier (18). This effect is largely a consequence of phosphate acting as a Brønsted-Lowry base catalyzing the loss of chloride from the primary chloroethylating species (19).

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Abbreviations

2,4-DNPH	2,4-dinitrophenylhydrazine (Brady's reagent)
4NBP	4-(4-nitrobenzyl)pyridine
90CE	1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine
BCNU (carmustine)	1,3-bis(2-chloroethyl)-1-nitrosourea
CNU	chloroethylnitrosoureas
DMSO	dimethyl sulfoxide
KS119	1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine
R-KS119W	1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(3-phospho-4-nitrophenyl)ethoxy]carbonyl]hydrazine

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Fig 1.

Panel A, Illustrates the reaction of a 'generic' alkylating electrophile with DNA, the standard colorimetric alkylating activity reagent 4NBP, and water. Reaction with water generates an alcohol which can be oxidized by alcohol oxidase to an aldehyde and then converted to a hydrazone by reaction with Brady's reagent. **Panel B**, Hydrazone quantification by HPLC. Authentic alcohol and aldehyde samples were analyzed by HPLC using the described method and exhibited a linear area under the curve to concentration relationship. The values are the means of at least 3 experiments $\pm$ SE. **Panel C**, Chemical scheme illustrating the activation of the investigational anticancer prodrugs (laromustine, KS119, R-KS119W) to generate 90CE, and potential fates of the hard oxophilic chloroethylating species derived from 90CE in the presence of DNA, phosphate (a representative Brønsted-Lowry base), and thiols. **Panel D**, The percentage molar yields of 2-chloroethanol from 90CE in the presence of inorganic phosphate and 1-thioglycerol. 90CE (1 mM) was allowed to decompose in 20 mM Tris-HCl, 1.0 mM EDTA buffer pH 7.4 at 37 °C in the presence of 0 – 20 mM 1-thioglycerol or organic phosphate and the yields of 2-chloroethanol determined by the described method. The values are the means of at least 3 experiments $\pm$ SE.

Table 1

Alcohol oxidation conditions, yields, and hydrazone HPLC identification

Alcohol/aldehyde	Alcohol oxidase u/mL [*] incubation	% Yield of aldehyde [†]	2,4-DNPH adduct elution time minutes	Activity assayed
Methanol	5 min 20° C 4 u/ml	84.7 ± 3.2	11.1 ^a	methylation
Ethanol	10 min 20° C 10 u/mL	89.3 ± 2.5	14.9 ^a	ethylation
Acetaldehyde	None	N/A	14.9 ^a	vinylation
Chloroethanol	30 min 40° C 10 u/mL	87.1 ± 3.2	16.3 ^a	chloroethylation
Ethylene glycol	1 hours 40° C 10u/ml	50.7 ± 2.3	9.1 ^b	hydroxyethylation
Glyoxal	None	N/A	9.1 ^b	control for ethylene glycol

^{*} MP Biomedicals LLC defined units: One unit will oxidize 1.0 μmole of ethanol to acetaldehyde per minute at pH 7.5 and 25°C.

[†] The % aldehyde yield from a 100 μM solution of the alcohol under the given conditions, calculation based upon a comparison with 100 μM of the corresponding authentic aldehyde. The values are the means of 3 experiments ± SE.

^a 30 mM potassium phosphate (pH 5.4), 52.5% acetonitrile, 0.8 mL/min.

^b 30 mM potassium phosphate (pH 5.4), 75% acetonitrile, 0.8 mL/min.