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INTERACTION OF THE AZIRIDINE MOIETY OF RSU-1069 WITH NUCLEOTIDES AND INORGANIC PHOSPHATE

IMPLICATIONS FOR ALKYLATION OF DNA

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Abstract—The aziridine moiety of RSU-1069 (1-(2-nitro-1-imidazolyl)-3-(1-aziridino-2-propanol)) alkylates inorganic phosphate and a range of nucleotides in aqueous solutions of pH 7.0. From the rate constants obtained and a study of the adducts formed it is demonstrated that phosphate is a major target on nucleotides at neutral pH, although additional sites may exist particularly on dGMP and dAMP. From the dependence of reactivity on pH and the influence of ionic strength, it is established that the protonated aziridine is the reactive species and that hydrolysis is insignificant when compared to the rate of phosphorylation. The reaction kinetics detailed in this study are discussed in terms of DNA alkylation and strand breakage effected by the aziridine moiety of RSU-1069.

RSU-1069 (Fig. 1), a hypoxic radiosensitizer [1] and chemopotentiator [2], has undergone phase I clinical trials. The aziridine moiety of RSU-1069 has been shown qualitatively to react with inorganic phosphate at pH 7.0 [3]. Since both parent and radiation reduced RSU-1069 are known to bind to DNA [3], it has been suggested that one mode of binding is via reaction of aziridine with the phosphate groups of DNA. One possible consequence of such an interaction may be the subsequent, rapid formation of single strand breaks at pH 7.0 [3, 4]. Both reduced and parent RSU-1069 cause strand breakage in plasmid DNA at pH 7.0, in contrast to the lack of strand breakage by RSU-1137 (Fig. 1) (the ring-opened aziridine analogue of RSU-1069) and misonidazole [3]. This strand breakage may be prevented by the addition of inorganic phosphate or 2-deoxyribose 5-phosphate.

Mitomycin C, a potent antitumour and antibiotic agent, and other aziridines are also known to alkylate inorganic phosphate [5–9]. However, mitomycin C requires low pH or reduction to activate the aziridine [5, 8]. The present study was therefore undertaken to establish the nature and reaction kinetics of the interaction of RSU-1069 with inorganic phosphate and both 3′- and 5′-nucleotides in order to assess possible differences in reactivity. Elucidation of the molecular aspects of the alkylating reactions of RSU-1069 with DNA components should assist in the identification of potential sites of attack for aziridine substituents on DNA.

MATERIALS AND METHODS

Chemicals. RSU-1069 was prepared as detailed previously [1]. Analytical grade material used to prepare phosphate buffers was purchased from BDH Chemicals Ltd. All other reagents were from Sigma and hplc columns from Hichrom Ltd.

Experimental. The interaction of RSU-1069 with inorganic phosphate, 2′-deoxyribonucleoside 5′-monophosphate (dNMP) and ribonucleoside 3′- and 5′-monophosphate (NMP) was followed using a Beckman 344 liquid chromatography system for hplc analysis. The following protocols were used:

(A) Isocratic chromatography was performed on a 5 μm spherisorb-CN column (25 × 0.5 cm^3 i.d.) with a mobile phase comprising 10% (v/v) methanol in 10 mmol/dm^3 KH_2PO_4, pH 3.0 at a flow rate of 1 cm^3/min. The detection of RSU-1069 and metabolites was performed at 310 nm and NMPs detected at 260 nm.

(B) Gradient elution chromatography was performed on a 5 μm spherisorb-ODS column (125 × 0.5 cm^3 i.d.) with a mobile phase comprising 10% (v/v) methanol in 50 mmol/dm^3 KH_2PO_4, pH 6.0, as the first eluent and 20% (v/v) methanol in 50 mmol/dm^3 KH_2PO_4, pH 6.0, as the second. A linear gradient up to 100% of the second eluent was passed through the column over 15 min at a flow rate of 1 cm^3/min. Isocratic chromatography used the second eluent. Detection of the products was as for A.

The kinetics of RSU-1069-P (the phosphorylated metabolite of RSU-1069) formation at 310 K were determined on interaction of RSU-1069 (10 mmol/dm^3) with phosphate in aqueous solution at pH 7.0 (unless stated otherwise). RSU-1069-P (Fig. 1) and RSU-1069 were assayed using hplc protocol A at given times after initiation of the reaction.

In order to determine the reactivity of RSU-1069 with the nucleotides, a competition method was used whereby the yield of RSU-1069-P was determined with different concentration ratios of phosphate : nucleotide. RSU-1069 (10 mmol/dm^3) in 200 mmol/dm^3 phosphate buffer (pH 7.0) containing 50–400 mmol/dm^3 dNMP or NMP were incubated at 310 K. Solutions were adjusted to pH 7.0 using perchloric acid. The pH remained unchanged during the reaction. Solutions were assayed using hplc protocol A.

RESULTS

The interaction of RSU-1069 with inorganic phosphate

RSU-1069 is known to react with inorganic phosphate to form the ionic product coded RSU-1069-P (structures in Fig. 1) [3]. RSU-1137 (Fig. 1), the aziridine ring-opened hydrolytic product is also formed together with two minor unknown products [3].

The rate of formation of RSU-1069-P and rate of loss of RSU-1069 on interaction of RSU-1069 (10 mmol/dm^3) with 0.05–0.5 mol/dm^3 phosphate at pH 7.0 was determined to be first-order and dependent upon the phosphate concentration as shown in Table 1 and Fig. 2, inset. On completion of the reaction <1% of RSU-1069 remains while the final RSU-1137 concentration varies from 13.1 (±0.6)% of the RSU-1069 lost at 0.05 mol/dm^3 phosphate to 8.5 (±0.2)% at 0.5 mol/dm^3 phosphate. The presence of an intercept on the plot of first-order rate constants against phosphate concentration (Fig. 2, inset) indicates that a reaction occurs which is independent of phosphate and has a rate constant of $\leq(7 \pm 2) \times 10^{-6}$/sec. This intercept is considered to represent the rate of hydrolysis of RSU-1069 to RSU-1137.

Since the dependence of rate constants on phosphate concentration is non-linear, the relationship of the ionic strength, calculated for each phosphate concentration, to the rate constant k_2 (k_1/[phosphate]) was determined for both RSU-1069-P formation and loss of RSU-1069 (Fig. 2) using the modified Debye–Brönsted relationship ($\log k = \log k_0 + 1.02 z_A z_B \mu^{1/2}/(1 + \mu^{1/2})$) [10]). The rate constants for loss of RSU-1069 and formation of RSU-1069-P are linear over the range of ionic strengths studied based upon the above relationship. The slope of the line (Fig. 2) was determined to be −1.9, a result in reasonable agreement with the theoretical slope of $1.02 z_{\text{RSU-1069}} + z_{HPO_4^{2-}} = -2.04$. The extrapolated value of the slope, 1×10^{-3} dm^3/mol/sec, yields k_2' at zero ionic strength. An attempt to repeat the above experiment with the concentration of RSU-1069 in excess of the phosphate failed because of the limiting solubility of the RSU-1069.

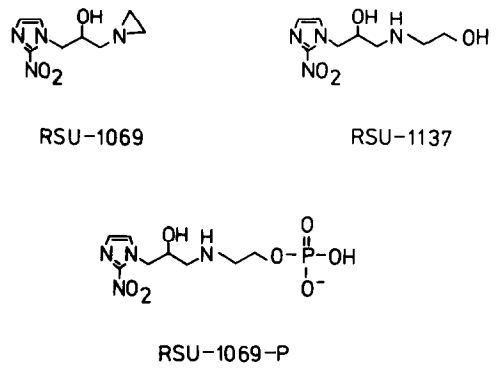


Fig. 1. Structures of RSU-1069, RSU-1069-P and RSU-1137.

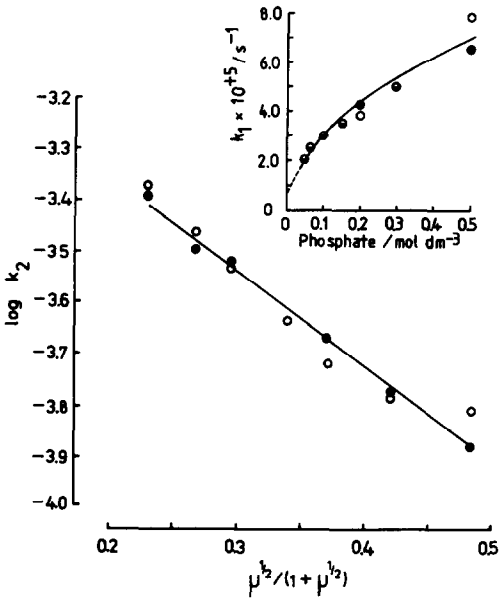


Fig. 2. Influence of ionic strength (μ) on the rate constant, k_2, for interaction of RSU-1069 with phosphate at pH 7.0. k_2 determined from formation of RSU-1069-P (○) and loss of RSU-1069 (●). Inset: dependence of k_1 on phosphate concentration. Formation of RSU-1069-P (○) and loss of RSU-1069 (●).

Table 1. First-order rate constants for RSU-1069-P formation and loss of RSU-1069

Phosphate (mol/dm^3)	Formation of RSU-1069-P		Loss of RSU-1069	
	$k_1 \times 10^{+5}$/sec	$k_2 \times 10^{+4}$ (dm^3/mol/sec)	$k_1 \times 10^{+5}$/sec	$k_2 \times 10^{+4}$ (dm^3/mol/sec)
0.05	2.11 ± 0.20	4.22 ± 0.40	2.02 ± 0.03	4.04 ± 0.06
0.075	2.58 ± 0.01	3.44 ± 0.01	2.40 ± 0.02	3.20 ± 0.03
0.10	2.92 ± 0.23	2.92 ± 0.23	3.02 ± 0.11	3.02 ± 0.11
0.15	3.44 ± 0.11	2.29 ± 0.07	3.45 ± 0.06	2.30 ± 0.04
0.20	3.80 ± 0.09	1.90 ± 0.05	4.28 ± 0.13	2.14 ± 0.07
0.30	4.93 ± 0.27	1.64 ± 0.09	5.10 ± 0.25	1.70 ± 0.08
0.50	7.81 ± 0.35	1.56 ± 0.07	6.56 ± 0.15	1.31 ± 0.03

Values are means of three determinations ± S.E.M.

* First-order plots were fitted using least squares linear regressive analysis and gave correlation coefficients >0.991.

† $k_2 = k_1$/[phosphate].

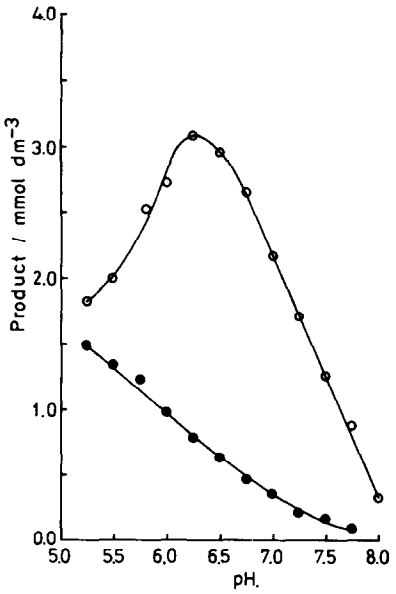


Fig. 3. The effect of pH on formation of RSU-1069-P (○) and RSU-1137 (●). Concentrations of RSU-1069-P and RSU-1137 determined using hplc protocol A after 3 hours incubation of RSU-1069 (10 mmol/dm^3) in 200 mmol/dm^3 phosphate at 310 K.

Influence of pH on RSU-1069-P formation

As shown in Fig. 3, the interaction of RSU-1069 (10 mmol/dm^3) with phosphate (200 mmol/dm^3), as expressed as the concentration of RSU-1069-P formed within 3 hr is dependent upon the pH, showing a maximum reactivity at pH 6.25. This maximum reactivity is between the pKa values of 6.0 [1] for the aziridine of RSU-1069 and 6.9 for phosphate ($HPO_4^{2-}/H_2PO_4^-$ at high ionic strength).

Interaction of RSU-1069-with nucleotides in the presence of phosphate

2' - Deoxyribonucleoside 5' - monophosphate (dNMP). In order to determine the reactivity of RSU-1069 (10 mmol/dm^3) towards the dNMPs, dAMP, dCMP, dGMP and dTMP, the concentration of RSU-1069-P in 200 mmol/dm^3 phosphate (Scheme 1) was determined in the absence and presence of known concentrations of dNMPs (50–400 mmol/dm^3) after completion of the reactions shown in Schemes 1 and 2.

$$\text{RSU-1069} + \text{PHOSPHATE} \xrightarrow{k_a} \text{RSU-1069-P}$$

Scheme 1

$$\text{RSU-1069} + \text{dNMP} \xrightarrow{k_b} \text{RSU-1069-dNMP}$$

Scheme 2

From initial experiments with 200 mmol/dm^3 phosphate in the presence and absence of 200 mmol/dm^3 dNMP it was established that the formation of RSU-1069-P is complete after 48 hr incubation at 310 K. It was also essential to establish that RSU-1069-P is not formed as a result of possible decomposition of the RSU-1069-nucleotide complex. This was tested by incubation of RSU-1069 (10 mmol/dm^3) in 400 mmol/dm^3 solutions of dNMP at pH 7.0 in the absence of inorganic phosphate. After 48 hours incubation at 310 K the solutions were assayed using hplc protocol A for RSU-1069-P and the concentration found to be less than the detection limit, thereby verifying that the RSU-1069-nucleotide product is stable to the formation of RSU-1069-P on this timescale. RSU-1137 is also formed with a yield similar to that produced under equivalent concentration conditions of inorganic phosphate (see above). The yield of RSU 1137 with 400 mmol/dm^3 dGMP is 0.40 mmol/dm^3, whereas the yield in the presence of 400 mmol/dm^3 dTMP is approximately twice at 0.75 mmol/dm^3 for an initial concentration of 10 mmol/dm^3 RSU-1069.

Based upon competition kinetics for reaction Schemes 1 and 2 and knowing that RSU-1069-P is produced only in 1, the following relationship represents the dependence of the yield of RSU-1069-P at various concentration ratios of phosphate : dNMP:

$$\frac{[\text{RSU-1069-P}]_\infty}{[\text{RSU-1069-P}]} = 1 + \frac{k_b[\text{dNMP}]}{k_a[\text{PHOSPHATE}]}$$

Scheme 3

$[\text{RSU-1069-P}]_\infty$ and [RSU-1069-P] represent the concentrations of RSU-1069-P in the absence and presence of dNMP respectively.

The relative reactivity of RSU-1069 towards the dNMPs were obtained from the slopes of lines shown in Fig. 4 which were based upon the relationship detailed in Scheme 3. The intercept of lines was shown to be approximately 1 for all four dNMPs (Fig. 4, Table 2) as predicted from Scheme 3. From the slopes, the rate constants for interaction of dNMP with RSU-1069 were determined taking k_2' to be

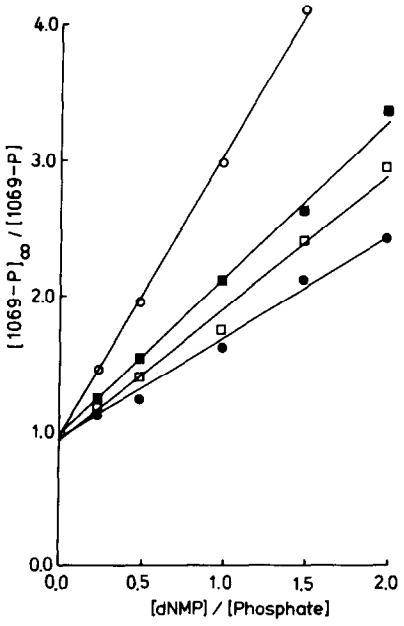


Fig. 4. Relative reactivity of RSU-1069 towards dAMP (■) dCMP (□), dGMP (○) and dTMP (●).

Table 2. Rate constants for interaction of nucleotides with RSU-1069 based upon competition kinetics

dNMP	Slope*	intcp	Rate constant $\times 10^{+3}$ (dm^3/mol/sec)
dAMP (4)	1.17 ± 0.04	0.97 ± 0.01	1.2
dCMP (4)	0.98 ± 0.03	0.92 ± 0.02	1.0
dGMP (4)	2.09 ± 0.03	0.96 ± 0.01	2.1
dTMP (3)	0.75 ± 0.10	0.95 ± 0.01	0.8
NMP			
3′ AMP (3)	1.25 ± 0.03	0.97 ± 0.02	1.3
5′ AMP (3)	0.84 ± 0.01	0.96 ± 0.01	0.8

Values are means ± S.E.M. with the number of determinations given in parenthesis
* Slopes taken from lines fitted using least square linear regression analysis. Lines gave correlation coefficients >0.993.

Table 3. RSU-1069-dNMP adducts formed at pH 7.0

	Retention time (min)			Percentage of total† identified products		
dNMP	A	B	C	A	B	C
dTMP	5.0	—	—	100	—	—
dCMP	5.8	6.5	—	92.9 ± 5.0	7.1 ± 4.3	—
dAMP	5.8	6.6	—	28.3 ± 0.5	71.7 ± 0.9	—
dGMP	5.4	6.4	6.8	47.8 ± 5.0	17.8 ± 5.0	34.3 ± 0.5

Products identified using hplc protocol A*. Values given are means of at least two determinations ± S.E.M.
* See Fig. 5 for spectral characteristics of the major products.
† Based on absorption at 310 nm.

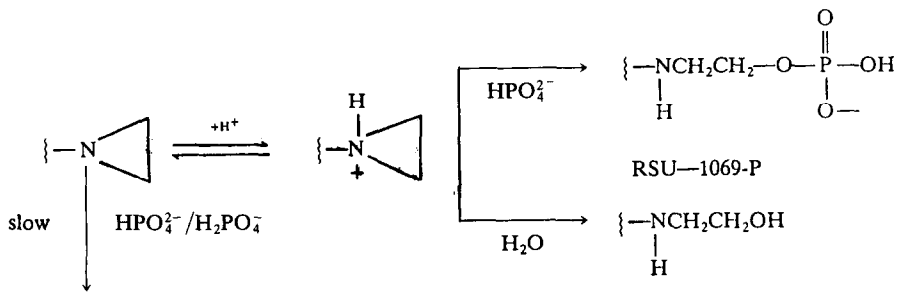
1.0×10^{-3} dm^3/mol/sec and are presented in Table 2. It is apparent that RSU-1069 is at least twice as reactive towards dGMP in comparison with other dNMPs and inorganic phosphate.

Adenosine 3′- and 5′-monophosphate (3′ AMP and 5′ AMP). In order to compare the reactivities of 3′- and 5′-NMPs with RSU-1069, competition kinetics as outlined above were performed using 3′- and 5′-AMP (50–400 mmol/dm^3). From the linear relationship based upon Scheme 3 the rate constants were determined and are presented in Table 2.

RSU-1069–dNMP adducts. RSU-1069 (10 mmol/dm^3) was incubated for 48 hr at 310 K in 400 mmol/dm^3 solutions of dNMP at pH 7.0. Aliquots were analysed using hplc protocol A and products identified as single peaks using dual wavelength facilities at 320 nm and 260 nm (Table 3). In the case of dTMP and dCMP only one major product was observed whereas with dAMP and dGMP more than one major product was apparent as shown in Table 3. The major product for each dNMP (dAMP B, dCMP A, dTMP A and dGMP A and C) was purified using hplc protocol A and shown to be a single peak with the isocratic and gradient elution chromatography of protocol B. Purified products were analysed using "on the fly" scanning and Fig. 5 shows scans of purified products eluted using hplc protocol A.

DISCUSSION

The interaction of RSU-1069 with inorganic phosphate results in the formation of an ionic salt product, RSU-1069-P and the following reaction scheme is proposed to account for the experimental observations presented.



Scheme 4

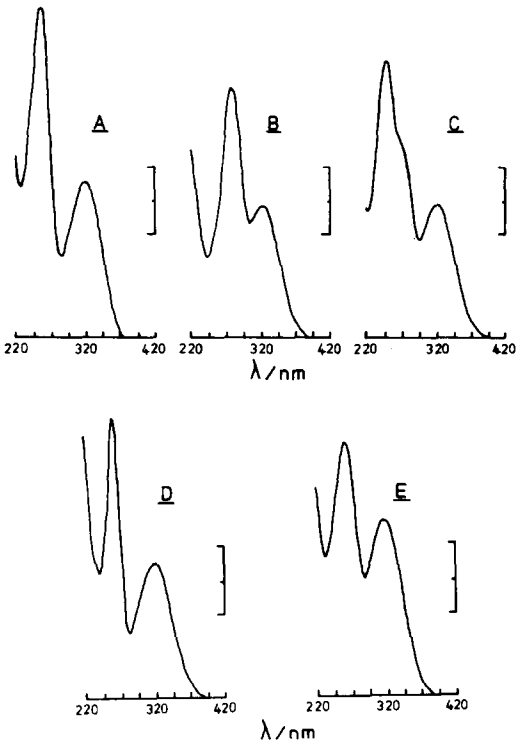


Fig. 5. Optical absorbance of major purified RSU=1069-dNMP adducts. A = dAMP B, B = dCMP A, C = dGMP A, D = dGMP C, E = dTMP A; scale bars represent 0.018, 0.020, 0.020, 0.020 and 0.016 absorbance units respectively.

Since it is well established that aziridines may interact by alkylation of inorganic phosphate [5–9] to produce the phosphorolytic ring-opened product, it is suggested that RSU-1069-P arises from alkylation of inorganic phosphate by RSU-1069. The analogous hydrolytic reaction [6], resulting in the formation of RSU-1137, also occurs (Scheme 4) and its formation observed on hplc analysis. The rate constant for the hydrolytic step to form RSU-1137 at pH 7 is estimated to be $\leq 7\ (\pm 2) \times 10^{-6}$/sec. This value is in reasonable agreement with that previously estimated from the stability of RSU-1069 in acetate buffer at pH 7 [3] and with those for other aziridines [6]. RSU-1137 may also arise from subsequent hydrolysis of RSU-1069-P with elimination of phosphate. However, this reaction is considered to be of minor importance under these conditions based upon the decreasing yields of RSU-1137 on increasing phosphate concentrations. This latter observation is consistent with the competition between hydrolysis and phosphorylation shown in reaction Scheme 4. Besides RSU-1137 and RSU-1069-P, two further unidentified peaks were observed on hplc analysis. These products may be formed on subsequent interactions of RSU-1069-P with RSU-1069 to produce polymeric products which are less ionic in character than RSU-1069-P. It should be stated that analogous products are not formed on interaction of nucleotides with RSU-1069.

From the similarity of the rate constants determined from either the loss of RSU-1069 or the formation of RSU-1069-P, it is inferred that the rate determining step is the loss of RSU-1069 and that if a transient intermediate is formed its subsequent decay to produce RSU-1069-P is not rate determining. Based upon the observed influence of the ionic strength on the rate constants with phosphate, it is inferred that the interaction between RSU-1069 and phosphate at pH 7 occurs preferentially between a dianion and a species with unit positive charge. It is therefore proposed that the major reactive species are the protonated aziridine of RSU-1069 and HPO_4^{2-}. It is known that aziridine reactivity is increased by the presence of H^+ as witnessed by the increase in yield of RSU-1137 on decreasing pH (Fig. 3). The interaction between the charged substrates as shown in Scheme 4 is also substantiated by the pH dependence of the yields of RSU-1069-P (see Fig. 3). In order to obtain a good fit of the experimental data in Fig. 3 the following reactions were used together with the pKa values of RSU-1069 [1] and phosphate.

$$(\text{RSU-1069})^+ + HPO_4^{2-} \xrightarrow{k_c} \text{RSU-1069-P}$$

$$(\text{RSU-1069})^+ + H_2PO_4^- \xrightarrow{k_d} \text{RSU-1069-P}$$

The best fit was obtained taking $k_d = 0.025\,k_c$, i.e. the rate constant with $H_2PO_4^-$ is ~40 times less than that with HPO_4^{2-}. A similar pH dependence for alkylation of inorganic phosphate by mitomycin C [p*K*a ~ 3.2 [11]] occurs at lower pH values [5], a finding consistent with these observations. The importance of the p*K*a of the aziridine group in governing its reactivity may be highlighted by the difference in reactivities of RSU-1069 and mitomycin C with phosphate [5] and the known biological inactivity of parent mitomycin C at pH 7, the p*K*a of mitomycin C being ~3 orders of magnitude less than that of RSU-1069 [1].

On interaction of RSU-1069 with nucleotides it is suggested that the reaction scheme is analogous to Scheme 4 whereby the phosphorolytic ring-opening reaction occurs via nucleophilic attack. In the case of the nucleotides, RSU-1137 is also formed the yield of which depends upon the nucleotide present. In fact the yield of RSU-1137 with dGMP is ~50% of that with dTMP consistent with the variation of the reactivities determined for RSU-1069. It is expected that the rate constants for interaction of the phosphate moiety of the nucleotides are similar since the p*K*a-values of the phosphate groups are similar [12]. Furthermore, the similarity of the rate constants for interaction of RSU-1069 with the 3′- and 5′-AMP and 5′-dAMP suggests that there is no preferential reactivity. It is therefore inferred from the observed variation of the rate constants with the nucleotides (Table 2) that alternative sites of attack exist as further substantiated from the product distribution (Table 3). If it is assumed that the rate constant for interaction of RSU-1069 with dTMP reflects attack at the phosphate moiety, then other major sites of attack may exist on the base moiety of dAMP and dGMP. It has previously been demonstrated [8, 13, 14] that mitomycin C alkylation may occur at N-2 and 0-6 of guanine and N-6 of adenine. On interaction with RSU-1069 one additional product occurs with dAMP and two with dGMP suggesting that alkylation by RSU-1069 may be analogous to that of mitomycin C.

In this study it has been possible to quantify and characterise the interactions of RSU-1069 with nucleotides and inorganic phosphate. Potential target sites on the DNA molecule are identified as the phosphate groups and the purine bases. RSU-1069 has been shown to cause significant strand breakage at pH 7.0 [3] whilst a post-incubation alkali treatment results in a significant increase in the yield of strand breaks over that observed at pH 7.0 (Silver *et al.*, unpublished data). Strand breakage as a consequence of alkylation of the purine bases may result from a further hydrolysis and/or depurination step. Furthermore, a hydrolytic step is required to give strand breakage following DNA-phosphate alkylation [15]. At present we are unable to ascertain which alkylated site(s) leads to strand breakage at pH 7.0 and which site(s) requires an alkali treatment to induce strand breakage. The existence of alkali-labile sites has been used to indicate the presence of alkylated bases [16]. It has also been inferred that alkali conditions decrease the stability of trialkylphosphates which are probably formed from RSU-1069 alkylation of DNA-phosphate. In order to identify the alkylated site(s) which results in the formation of breaks at pH 7.0 and alkali-induced breaks, experiments with DNA are presently being conducted to establish the individual alkylating processes which result in strand breakage.

The observations presented in this paper are applicable to other aziridine containing substituents and may assist in the interpretation of their mode of action in terms of DNA damage. The identification of these molecular processes may lead to the development of more efficient alkylating agents.

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