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INTERACTION OF RSU 1069 AND 1137 WITH DNA *IN VITRO*

BIOLOGICAL IMPLICATIONS AND MECHANISTIC ASPECTS

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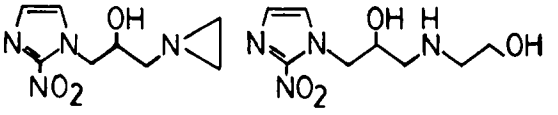
Abstract—We have examined the capacity of the nitroimidazole aziridine antitumour drug RSU 1069 to react with DNA *in vitro* in order to get a better understanding of its mechanism of action. Moreover, we have utilized biologically active ΦX174 DNA to investigate the biological relevance of the chemical DNA modification induced by the drug. Incubation of RSU 1069 in the presence of single-stranded ΦX174 DNA resulted in extensive inactivation of the DNA, which is dependent on the concentration of drug and temperature. Only about 2% of the inactivating damage can be attributed to strand breakage. The main damage most probably consists of base damage, of which a part is non-lethal and alkali-labile which in turn can be converted into lethal lesion and subsequently into a break applying a post-incubation alkali treatment. Furthermore, from the dependence of the inactivation and also the formation of breaks on pH and ionic strength, it is concluded that the reaction most probably takes place between a protonated RSU 1069 and a negative DNA coil and that the damage pattern reflects the difference in reactivity of RSU 1069 with the phosphate groups and the bases in DNA. Comparison between RSU 1069 and its ring-open hydrolysis product RSU 1137 revealed that (lethal) damage induced in the DNA must be ascribed to the alkylating properties of the aziridine moiety.

In a number of patients, receiving treatment with radiation, there is a failure to control the tumour because of the presence of a radiation-resistant hypoxic cell fraction, especially in solid tumours. Nitroimidazole radiosensitizers are being extensively investigated because of their specific radiosensitizing property for hypoxic cells and additional cytotoxicity.

In general the data obtained in the clinic with these nitroimidazole radiosensitizers, mostly with misonidazole, were disappointing because of dose-limiting side effects such as neurotoxicity, although in a few cases benefit has been reported [1]. Recently some compounds are developed with sensitizing efficiencies much higher than would be predicted from their electron-affinities. One such compound is RSU 1069 which contains, besides a nitro-group, an aziridine moiety and therefore has alkylating properties. This drug is considerably more efficient than misonidazole as a hypoxic radiosensitizer and chemopotentiator [2, 3]. Moreover, increase in the efficacy of RSU 1069 has been demonstrated *in vivo* with vasoactive drugs [4, 5]. Since these nitroimidazole drugs are supposed to act against DNA as the major cellular target it is important for a good understanding of their mechanism of action to investigate the interaction of the drug with DNA directly. Previous studies with deoxynucleotides, inorganic phosphate and plasmid DNAs have indicated that in case of RSU 1069 reactions with the phosphate and purine bases in the DNA are likely to occur, leading to strand breakage and alkali-labile sites [6–9]. In these reactions, most probably, the aziridine moiety of the drug is involved. Although some reports stress the importance of double-strand breaks in cellular DNA and suggest a possible role of bulky adducts [10–13], direct evidence about the biological significance of the chemical DNA modifications brought about by RSU 1069 is hardly available. Therefore, we investigated the action of RSU 1069 and its aziridine ring-opened hydrolysis product RSU 1137, which under certain circumstances will be a major byproduct [7] (chemical structures are given in Fig. 1), using biologically active DNA from the bacteriophage ΦX174. This single-stranded DNA enables us to examine the influence of a specific chemical DNA modification on the biological functioning of the DNA, thereby providing evidence about its biological relevance. Our results show that RSU 1069 most probably inactivates single-stranded ΦX174 DNA mainly through alkylation of the DNA bases. (Alkali-labile) breaks contribute only for a small part to the lethal damage. This work has been published in part in abstract form [14, 15].



RSU_1069 RSU_1137

Fig. 1. Chemical structures of RSU 1069 and RSU 1137.

MATERIALS AND METHODS

Drugs and chemicals. RSU 1069 and RSU 1137 were obtained as a gift from Drs Stratford and Jenkins (MRC Radiobiology Unit, Chilton, Didcot, U.K.) and provided by Prof. Edwards (Polytechnic of East London, Romford Road, London, U.K.). RSU concentrations were determined spectrophotometrically at 325 nm ($\varepsilon = 7000$). All other chemicals were of analytical grade and used as supplied. All solutions were prepared in triple distilled water. Sonication was used to dissolve the RSU drugs.

Exposure of DNA to RSU 1069 *and RSU* 1137. Usually single-stranded ΦX174 DNA (1–3 μg/mL) and RSU 1069 or 1137 (3×10^{-4} mol/dm^3) were allowed to react in the dark for increasing periods of time, in 10^{-3} mol/dm^3 sodium phosphate buffer at pH 7.2 ± 0.1 and 37° with an additional 10^{-2} mol/dm^3 sodium chloride present in the solution to maintain a constant ionic strength, unless specified otherwise. Variation in ionic strength was obtained by adjusting the NaCl concentration. At various time intervals samples were taken and diluted at least 100-fold with 5×10^{-2} mol/dm^3 Tris–HCl pH 8.0 on ice to stop the reaction, and used as such for the transfection assay. For analysis on sucrose gradients reactions were stopped by 2 mol/dm^3 NaCl or NaH_2PO_4 (final concn. 0.2 mol/dm^3) followed by storage on melting ice until the analysis. No correction has to be made for effects due to pH, temperature or ionic strength themselves (without drug). All effects observed in the experiments are independent of the DNA concentration, although of course the absolute amounts of damage differ.

Post-incubation alkali treatment. Following exposure to the drug for given time intervals the DNA was assayed for strand breakage and biological inactivation after an alkali treatment. Before the alkali treatment 2 mol/dm^3 NaCl/NaH_2PO_4 (final concn. 0.2 mol/dm^3) was added to prevent further reaction of the RSU drug with DNA. Subsequently 0.5 mol/dm^3 NaOH was added to the reaction mixtures (final pH ~12.5) followed by incubation at 37°. At various time intervals the samples were neutralized by addition of 0.5 mol/dm^3 HCl in 10^{-2} mol/dm^3 phosphate buffer and then immediately placed on ice awaiting further analysis.

DNA and Transfection assay. The methods of preparing single-stranded DNA from the bacteriophage ΦX174 has been described by Blok *et al.* [16]. The DNA concentrations were determined at 260 nm ($\varepsilon = 8200$). The biological activity of the DNA was measured using an *Escherichia coli* (AB 1157) transfection assay essentially described before [16, 17]. All samples tested were diluted at least 100-fold with 5×10^2 mol/dm^3 Tris–HCl pH 8.0 prior to the transfection assay. Survival curves were obtained by plotting the logarithm of the fraction of surviving DNA (measured as bacteriophages) against time.

Determination of strand breaks. Linear sucrose gradients (5–40% w/v in 10^{-2} mol/dm^3 NaCl + 10^{-4} mol/dm^3 EDTA + 10^{-3} mol/dm^3 phosphate buffer pH 7.2) were prepared in cellulose nitrate tubes. Incubation mixture (450 μL) containing 1.4 μg of DNA was layered on top of the gradient. Centrifugation was performed at 15° and 36,000 rpm for about 19 hr in a Beckman SW41 rotor. After centrifugation the gradients were analysed [18]. From the sedimentation pattern the amount of unbroken molecules can be derived. From the exponential decrease of this amount the time necessary to introduce on the average one break per DNA molecule (T_{37} break) can be concluded).

RESULTS

DNA inactivation by RSU 1069

To get an impression of the DNA inactivating potential of RSU 1069 different concentrations of the drug were allowed to react with single-stranded ΦX174 DNA for increasing periods of time. The inactivation could be described over at least 4–5 decades by exponential survival curves and thus the T_{37} value, the time at which 37% of the DNA molecules are still active is a measure for on the average one lethal hit per DNA molecule (Fig. 2a). Furthermore, the amount of lethal damage per time per DNA molecule ($1/T_{37}$) appears to be, as may be expected, linearly dependent on the drug concentration (slope = 1 on a double log plot, Fig. 2b). The same applies to the induction of single-strand breaks (data not shown).

The effect of RSU 1069 *as a function of* pH

Silver and O'Neill [7] showed that RSU 1069 reacts with inorganic phosphate and that this reaction is dependent on pH. Furthermore, it has been suggested that strand break formation in DNA may be due to reactions of RSU 1069 with the phosphates in the DNA backbone [6–8]. Therefore, we looked at the pH dependence on both the induction of strand breaks and biological inactivation of DNA by RSU 1069. As shown in Fig. 3a the formation of breaks in single-stranded ΦX174 DNA appears to be dependent on pH with a maximum effect at pH ≤ 6.2, indicating that break formation may be ascribed to reaction with phosphates in the DNA. This effect parallels the pH dependence seen for the inactivation of the DNA (Fig. 3b). However, from their respective T_{37} values at the given pH values the contribution of breaks to lethality is calculated to be low (1.6 ± 0.7%), for the whole pH range, showing that the observed DNA inactivation is for the most part due to other reactions than with the phosphate groups of the DNA. To be certain that the observed pH effects are not due to the changing $HPO_4^{2-}/H_2PO_4^-$ ratio, which is of course influenced by pH, the phosphate has been left out in an otherwise unchanged reaction solution. Although the absolute amounts of damage differ (more damage without phosphate) the pH dependence of the RSU 1069 reaction with DNA remains unaltered. On the other hand raising the phosphate concentration from 10^{-3} to 10^{-2} mol/dm^3 further protected the DNA. Probably DNA and phosphate simply compete for RSU 1069.

Influence of temperature

It is well known that the rate of a reaction depends on temperature. As can be seen in Fig. 4 this also holds for the reaction of RSU 1069 with DNA. Fitting the data with a least squares analysis an increase in the rate of inactivation was determined

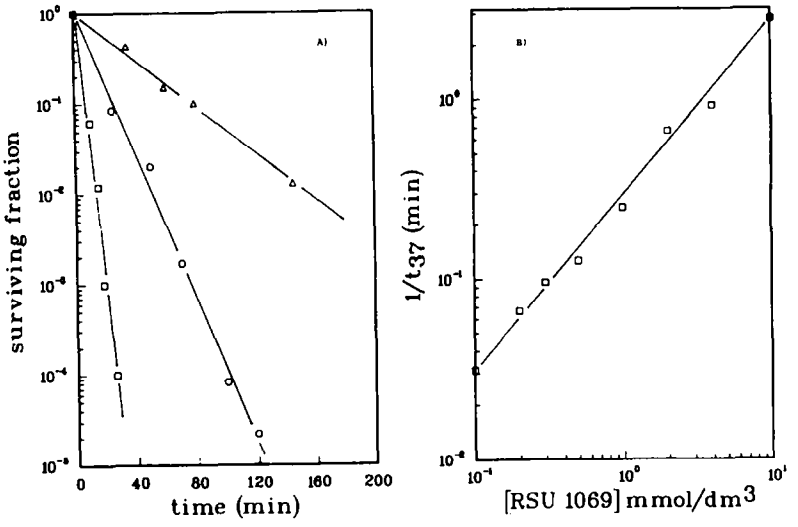


Fig. 2. The effect of RSU 1069 on the biological activity of single stranded ΦX174 DNA (1 μg/mL) dissolved in 1 mmol/dm³ phosphate buffer + 10 mmol/dm³ NaCl pH 7.2 and incubated at 37° with different concentrations of RSU 1069 for increasing periods of time. (a) Survival curves: (□) 1 mmol/dm³ RSU 1069, (○) 0.3 mmol/dm³ RSU 1069, (△) 0.1 mmol/dm³ RSU 1069. (b) Dependence on RSU concentration. The T_{37} values are calculated from the slope of complete survival curves as presented in (a). Each point represents the mean value of at least two independent determinations.

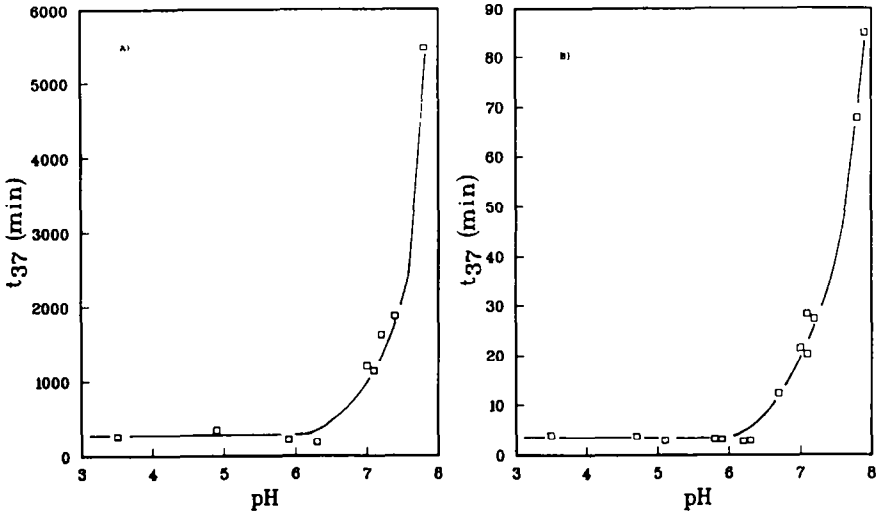


Fig. 3. Effect of pH on the induction of breaks (a) and biological inactivation (b) of single-stranded ΦX174 DNA (3 μg/ml) by RSU 1069 (0.3 mmol/dm³). Incubations were performed in 1 mmol/dm³ phosphate buffer + 10 mmol/dm³ NaCl at 37°, pH values >7.5 were adjusted by NaOH and <5.3 by HCl, T_{37} values are obtained as explained in Fig. 2 and for the induction of breaks in a similar way.

to be 0.2 ± 0.02/°C. The same applies to the induction of breaks. The activation energy for the reaction of RSU 1069 and DNA has not been calculated, because of the complexity of reactions involved.

Ionic strength

As can be inferred from the work with inorganic phosphate [7] it can be expected that electrostatic forces will be involved in the reaction of RSU 1069 with DNA. Indeed interaction of RSU 1069 is shown to be affected by the ionic composition of the reaction solution. Increasing concentrations of sodium chloride inhibited the inactivation of the DNA (Fig. 5), although no complete prevention could be accomplished. Furthermore, the data suggest that the

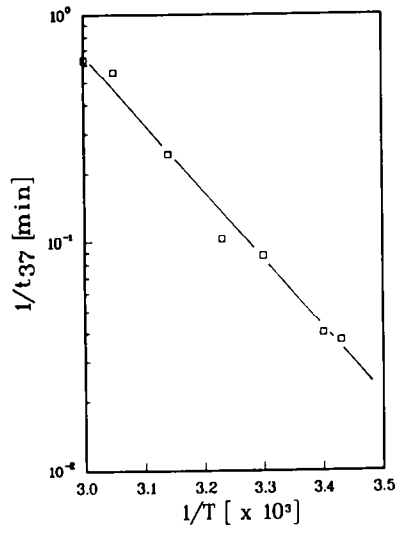


Fig. 4. Influence of temperature on the interaction of RSU 1069 with DNA. One μg/mL single-stranded ΦX174 DNA together with 0.3 mmol/dm³ RSU 1069 were incubated in 1 mmol/dm³ phosphate buffer pH 7.2 + 10 mmol/dm³ NaCl. T_{37} values are obtained as explained in Fig. 2.

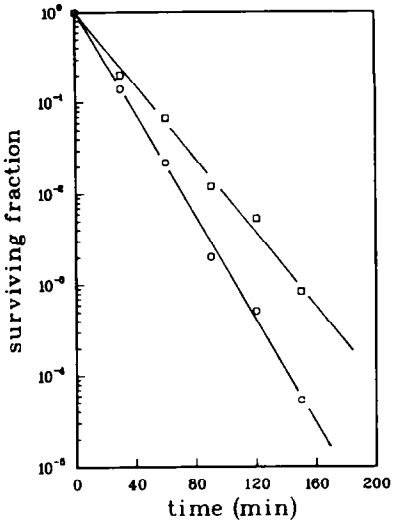


Fig. 6. The effect of a post-incubation alkali treatment on the survival of the biological activity of single-stranded ΦX174 DNA (3 μg/mL) incubated with 0.3 mmol/dm³ RSU 1069 at 37° dissolved in 1 mmol/dm³ + 10 mmol/dm³ NaCl. (□) No post-incubation alkali treatment, (○) 60 min post-incubation alkali treatment (pH 12.5) at 37°.

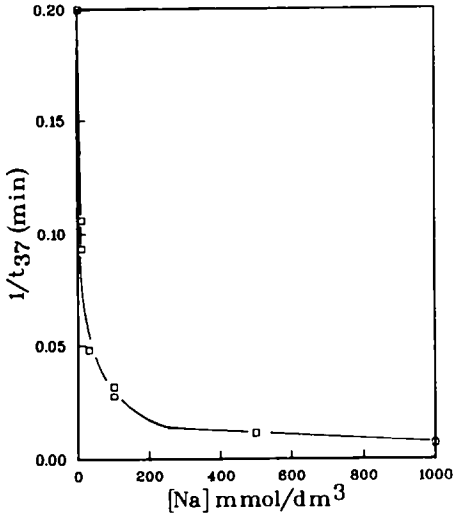


Fig. 5. Effect of the ionic composition of the reaction solution on the inactivation of single-stranded ΦX174 DNA One μg/mL DNA was incubated with 0.3 mmol/dm³ RSU 1069 and different concentrations of NaCl in 1 mmol/dm³ phosphate buffer pH 7.2 at 37°. The T_{37} values are obtained from complete survival curves.

inhibition of the inactivation as reflected by the $1/T_{37}$ values depends on the square of the Na^+ concentration. The same dependence has been observed for the formation of single-strand breaks. So the contribution of breaks to lethality (about 2%; see section on pH) is not affected by the ionic strength and remains constant. Furthermore, the effects of the ionic strength for both inactivation and breaks are independent of the temperature (18–45°) at which the reactions take place, within the time course applied.

Detection of alkali-labile sites

To reveal the presence of alkali-labile breaks in single-stranded ΦX174 DNA which has been found in double-stranded plasmid DNA [6, 8] we applied a post-incubation alkali treatment. Moreover, alkali-labile sites may be indicative for alkylated DNA bases, since it is known that this type of damage can lead to spontaneous or alkali catalysed breakage of the *N*-glycosidic bond, generating AP-sites [19, 20], which in their turn can be converted into breaks by alkali.

As a result of the alkali treatment a decrease in survival of the DNA is observed (Fig. 6), revealing the existence of non-lethal damage.

Also the number of single-strand breaks is increased considerably (max. ~15 times). Still most (~80%) of the (lethal) damage is not associated with a strand break. Furthermore, the results show that the decrease in survival/increase in inactivation already starts before a significant increase in breaks can be observed. However, the fact that the same maximum is found for both strand break formation and inactivation suggest a close correlation between the formation of alkali-labile breaks and extra inactivation.

The effect of RSU 1137

Under all conditions described above for RSU 1069, no DNA inactivation or the introduction of strand breaks were observed upon incubation of single-stranded ΦX174 DNA with RSU 1137.

Apparently, the inactivation by RSU 1069 is mainly due to the reaction of the aziridine moiety with DNA.

DISCUSSION

In this paper we investigated the biological consequences of the interaction between the aziridine nitroimidazole RSU 1069 and single-stranded DNA. Data from the literature obtained with DNA components suggest that both inorganic phosphate and the DNA bases (in particular G;A) are susceptible to reaction with RSU 1069 [7]. Furthermore, by the use of double-stranded plasmid DNAs (alkali-labile) single-strand breaks could be observed [6–9].

The results presented show clearly that the reaction between RSU 1069 and the DNA leads to damage which is lethal to the DNA. However, only a small part (~2%) of this damage consists of single-strand breaks, which are always lethal in single-stranded ΦX174 DNA [21]. If these breaks can be attributed to the reaction of RSU 1069 with the phosphate groups of the DNA backbone then other major sites of attack must exist in DNA, possibly the purines. It has been suggested that reaction with the bases will lead to labile sites, which can be detected as a break by applying an alkali treatment [7–9]. Indeed alkali-labile sites are found in single-stranded ΦX174 DNA after reaction with RSU 1069. Moreover, the alkali treatment revealed the existence of non-lethal damage, which requires alkali to become lethal (see Fig. 6, 7). It is known that alkylated DNA bases can be removed by an incubation generating AP sites [19, 20, 22]. The DNA chain is susceptible to (alkaline) hydrolysis at the AP sites, resulting in a chain break [19–21]. If we assume that the increase in inactivation by the alkali treatment is the result of the formation of AP sites, which are known to be lethal in single-stranded ΦX174 DNA [24, 25] than subsequently also breaks will be formed by the treatment. This is illustrated by the reaction pattern of the formation of alkali-breaks (Fig. 7). After a lag time probably due to the formation of susceptible sites accompanied by an increase in inactivation, the pattern resembles the one found for the conversion of apurinic sites, introduced by heat/acid treatment into breaks [24, 25] and therefore supports the assumption about introduction of AP sites. This will be in line too with the observation that the contribution of breaks to lethality remains constant by raising the temperature from 18–45° during the incubation with RSU 1069. Under these experimental circumstances no significant breakage of AP sites by these temperatures has to be expected in contrast to, for example, OH radical induced labile DNA damage [25]. Further substantial support comes from the fact that the maximum increase in the amount of breaks equals (within the limits of error) the increase in biological inactivation as can be expected if the non-lethal damage eventually becomes a break by the alkali treatment. Concerning the nature of the non-lethal damage: the suggestion of an altered DNA base through alkylation by the RSU 1069 may be made leading to labilisation of the *N*-glycocidic bond. So we propose the following reaction scheme:

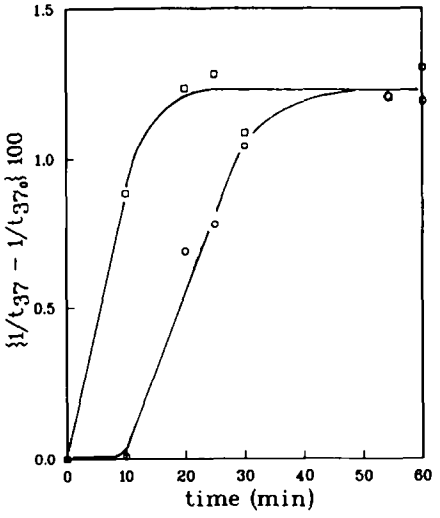
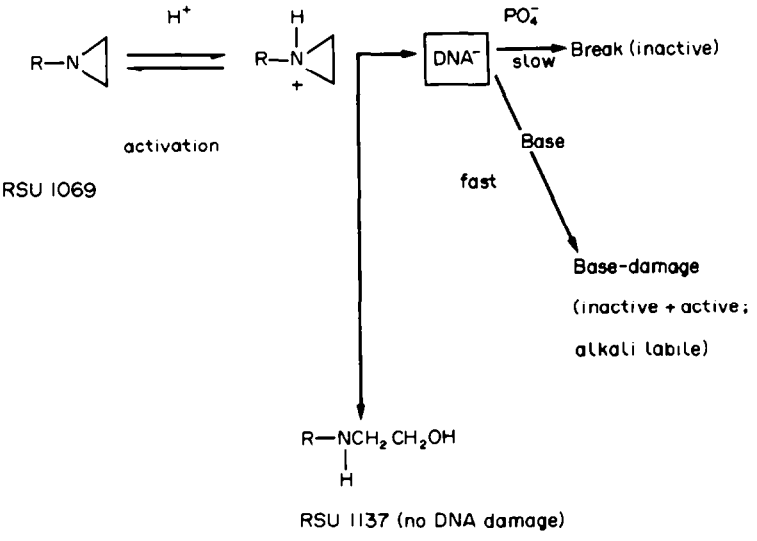


Fig. 7. Increase in the amount of damage ($1/T_{37}-1/T_{37_0}$) by post-incubation treatment as a function of time of this treatment. T_{37_0} and T_{37} values are obtained from survival or break induction curves before and after an additional alkali treatment, respectively. Data derived from similar experiments as given in Fig. 6. (□) Increase in biological inactivation, (○) increase in single-strand breaks.

alkylated DNA bases (still biologically active) → AP sites (inactive) → breaks (inactive)

Non-lethal damage is not uncommon in single-stranded ΦX174 DNA [24–30]. Also damages have been described with a similar sequence of reactions as given above [28, 29]. Apparently single-stranded DNA can sustain an appreciable amount of damage without affecting its biological activity. Although the proposed scheme seems rather likely we cannot exclude completely that alkylation of the DNA-phosphate is responsible to some extent for the observed effects, because according to Shooter and Merrifield [31] a hydrolytic step seems to be required to give strand breakage at such sites. On the other hand O'Neill *et al.* [8] expect that the phosphotriester formed with RSU 1069 is unstable already at neutral pH and therefore no alkali should be required for the formation of a strand break. In conclusion we may say: although clearly (alkali-labile) breaks are formed they account only for a small part to the DNA damage (~20%). Because sugar damage is rather unlikely, as can be inferred from the work by Silver *et al.* [6], the majority of the lethal damage must therefore consist of some form of base damage, probably through alkylation of preferentially the purine bases [7] and analogous to that of mitomycin C [32].

So far we discussed the biological implications of the reaction of RSU 1069 with DNA leading to several types of damage. In order to get more information about the mechanism of action on the DNA level we looked at the effect of ionic strength; the higher the ionic strength the less inactivating damage. This observation cannot be explained by shrinking of the DNA coil with increasing ionic



Scheme 1.

strength thereby influencing the reactivity of the DNA [18, 33]. Here the dependence on the ionic strength is much more pronounced and has a different pattern. However, electrostatic forces may be involved in another way. Possibly reactions between a positive charged RSU 1069, localized on the aziridine moiety, and the negative DNA coil play an important role as proposed for the reaction between HPO_4^{2-} and RSU 1069 [7]. In DNA phosphate is in the singly negative charged form being less reactive than the doubly negative charged form or deoxynucleotides [7]. This is possibly witnessed by the lack of a substantial amount of strand breaks (~2%) in the DNA. Furthermore, the contribution of breaks to lethality is independent of the ionic strength suggesting that the protonated RSU 1069 is attracted to the DNA by its negative forces, but the damage pattern reflects the difference in reactivity between the bases and phosphate in DNA with RSU 1069. This is substantiated by the similar pH effects on the DNA damages (inactivation, strand breaks). Although the reaction with phosphate is of minor importance in single-stranded DNA there is a resemblance with the pH effect on the reaction of RSU 1069 with inorganic phosphate [7] indicating again that activation (protonation) of the aziridine group of RSU 1069 is the determining reaction. Moreover, the absence of any effect of the presence of phosphate in the solution on the pH dependence of the DNA damage indicates that the ratio $HPO_4^{2-}/H_2PO_4^-$ does not determine this pH effect. Phosphate in the solution merely acts as a competitor with DNA for RSU 1069, in line to the effects described by Silver and O'Neill [7].

Activation of RSU 1069 is accompanied by a hydrolytic reaction resulting in a pH dependent formation of the ring-open product RSU 1137: more RSU 1137 is formed at decreasing pH [7]. For that reason we examined the possible contribution of RSU 1137 to the above observed effects. Since RSU 1137 is unable to induce any form of damage in single-stranded ΦX174 DNA, which corroborates the absence of effects by misonidazole (unpublished data), the damage seen after incubation with RSU 1069 can be fully attributed to the aziridine function of the RSU drug in line with the observations made with ethanol-aziridene [6]. However, RSU 1137 might explain to some extent the levelling off seen at low pH (≤6.2) of the damage, because here RSU 1137 will be formed in competition with activated RSU 1069 to react with DNA.

Scheme 1, inspired by the one given for the reaction between inorganic phosphate and RSU 1069 [7], is proposed to explain the presented results.

In conclusion, RSU 1069 inactivates single-stranded ΦX174 DNA through the aziridine moiety. Contribution of single-strand breaks to the DNA damage is very small. Alkylation of the DNA bases seems to be important for inactivation. Only part of the damage can be recognized as alkali-labile and therefore can be not more than indicative for base damage.

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