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The enhancement of the after effect of ionizing radiation by a cytotoxic methylhydrazine derivative

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Abstract

The cytotoxic methylhydrazine derivative *N*-isopropyl-*p*-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan®) degrades DNA in solution by a similar mechanism to ionizing radiation. The combined treatment of DNA with ionizing radiation and with Natulan® causes more double breaks of DNA molecules than would be expected from a simple summation of the effects of radiation and of the cytotoxic agent. The synergism between ionizing radiation and Natulan® is demonstrated by means of dose–effect curves. To obtain the greatest synergistic effect Natulan® has to be added immediately after termination of irradiation. No synergism can be observed between irradiation and a cytotoxic compound of the alkylating type (''Nitrogen Mustard''). It is suggested that the unstable peroxide products formed during irradiation of DNA are responsible for the synergism between irradiation and Natulan®.

Résumé

Un dérivé cytotoxique de la méthylhydrazine, le chlorhydrate de *N*-isopropyl-*p*-(2-méthylhydrazinométhyl)benzamide (Natulan®, dégrade l' ADN en solution par un mécanisme comparable à celui des radiations ionisantes. L'action combiniée d'une radiation ionisante et du Natulan® provoque des doubles ruptures des chaînes ADN en plus grand nombre que la somme de leurs effets isolés. Cette synergie est démontrée par la relation dose–effet. On obtient l'effet synergique maximum en ajoutant le Natulan® sitôt après cessation de l'irradiation. Aucune synergie n'a été observée par combinaison de radiations et d'un composé cytotoxique du type agent alcoylant (''moutarde azotée''). On suppose que l'effet synergique observé avec les radiations et le Natulan® est dû aux peroxydes in-stables,formés durant l'irradiation de l'ADN.

Zusammenfassung

Die cytotoxische Methylhydrazin-Verbindung *N*-Isopropyl-*p*-(2-methylhydrazinomethyl)benzamid-hydrochlorid (Natulan®) bewirkt ähnlich wie ionisierende Strahlen einen Abbau von Desoxyribonucleinsäure *in vitro*. Bei Kombination von Natulan mil ionisierender Strahlung is der Effekt grösser als die Summe der Einzeleffekte. Der Synergismus zwischen ionisierender Strahlung and Natulan kann anhand von Dosis-Wirkungs-Kurven nachgewiesen werden. Der stärkste Effekt wird erhalten,wenn Natulan® unmittelbar nach Beendigung der Bestrahlung zugesetzt wird. Das alkylier-ende Cytostaticum Methyl-bis-β-chloraethylamin-hydrochlorid (''Nitrogen Mustard'') Zeigt im erwähnten Modellsystem keinen Synergismus mit ionisierender Strahlung. Der beobachtete Synergismus wird als Folge der Bildung instabiler peroxidischer Produkte während der Bestrahlung erklärt.

1. Introduction

Evidence has been published recently for the synergistic effect between the cytotoxic methylhydrazine derivative *N*-isopropyl-*p*-(2-methylhydrazino-methyl) benzamide hydrochloride (Natulan®) and ionizing radiation on DNA degradation, the greatest effect being obtained when the compound is added immediately after termination of irradiation [1]. It was demonstrated that this combined action causes more double breaks in

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the DNA molecule than would be expected from a simple summation of the effects of radiation and of the cytotoxic agent. It had been shown previously that hydrogen peroxide, which is formed [2,3] and activated [4,5] during autoxidation of Natulan, is responsible for the DNA degradation by the latter. Interestingly enough no synergistic effect has been obtained when Natulan was added *before* the irradiation. This is probably due to competitive radioprotective reactions of Natulan *during* irradiation resulting from its reducing power, e.g., "repair" of DNA radicals. Similar effects were observed by Butler *et al.* [6,7] in the case of cysteine which protects DNA when present during irradiation but accelerates the rate at which the after effect occurs when added immediately after irradiation.

In the following, experimental results will be presented which show that the synergism between radiation and Natulan is much more pronounced than the synergism between radiation and hydrogen peroxide. It will further be demonstrated that the synergism between radiation and Natulan decreases with increasing time interval between the end of irradiation and the addition of Natulan, whereas in the case of nitrogen mustard the extent of DNA degradation is not dependent on the time interval between the end of irradiation and the addition of the compound. Finally dose–effect curves are presented which clearly prove the synergism between Natulan and ionizing radiation.

2. Methods

Experimental details have been described before [1–4]. The results presented in the following were obtained with sodium deoxyribonucleate (DNA) prepared from calf thymus glands. The average molecular weight of the preparation used was about 3 millions. A 0.05% solution of DNA in 1/30 molar phosphate buffer was prepared containing 10% sodium chloride to stabilize the double helix structure of the DNA [8]. Sodium pyrophosphate 0.002 mol/l were added to remove iron ions [9]. For the irradiation experiments a cobalt-60 γ-ray source of 112 c was used [10]. Ten millilitres of the solutions in cylindrical flasks were placed at such a distance from the radiation source that they received the various doses required within 1 hr. Irradiation was carried out at room temperature. After termination of the irradiation the samples were stored at 37 °C. The samples to which the compounds were added without preceding irradiation were treated in the same manner. The cytotoxic compounds Natulan® [11–13], and the nitrogen mustard methyl-bis(β-chloroethyl)-amine hydrochloride were added in solid form. Hydrogen peroxide was added as 30% aqueous solution (*pro analysi*, Merck Darmstadt). All experiments were carried out in air.

The degradation of the DNA was followed by viscosity measurements and by the determination of sedimentation constants. The viscosities were determined in an Ostwald type viscometer under a sheer stress of about 200–500 sec^{-1} at 37 °C. Specific viscosities were calculated according to Staudinger and Heuer [14]. The sedimentation constants of the DNA before and after degradation were determined with a Spinco analytical ultracentrifuge type E at 20 °C in the concentration range between 0.02% and 0.05% and extrapolated to the concentration limes zero.

3. Results

3.1. Differences between Natulan and hydrogen peroxide with respect to synergism with ionizing radiation

Butler *et al.* [6,7] have shown that DNA irradiated with X-rays in solution is more susceptible to hydrogen peroxide than unirradiated samples when hydrogen peroxide is added not later than about 20 hr after irradiation. Natulan yields about the equimolar amount of hydrogen peroxide in aqueous solution in the presence of air as a result of its autoxidation [2,3]. We therefore compared the effect of equimolar amounts of Natulan and of hydrogen peroxide on irradiated DNA solutions. The compounds were added immediately after termination of irradiation. In Fig. 1 the results of these experiments are presented. They show that the synergism between Natulan and irradiation is much more pronounced than between hydrogen peroxide and irradia-

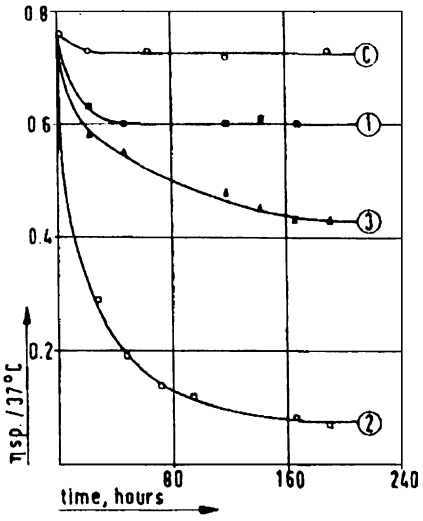

Fig. 1. Change in the specific viscosity of a 0.05% solution of DNA after the following treatments: (C), Control (without treatment); (1) irradiation with 11,000 rad; (2) irradiation with 11,000 rad and addition of 0.0005 mol/l *N*-isopropyl-*p*-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan®), immediately after termination of irradiation; (3) irradiation with 11,000 rad and addition of 0.0005 mol/l hydrogen peroxide *immediately* after termination of irradiation.

tion. The synergism between irradiation and Natulan can therefore not be explained simply as a result of the hydrogen peroxide formation by the latter compound.

3.2. Influence of the time interval between irradiation and addition of Natulan on the degradation of DNA

Curve 1 of Fig. 2 shows the reduction in viscosity of the DNA solution during 1 hr of irradiation with 11,000 rad which is followed by a further but rather slow decrease of viscosity due to the "after effect" [6,7,15–29]. The curves 2–5 present the comparatively rapid reduction in viscosity which is induced by the addition of 0.0005 mol/l Natulan® to the irradiated solution. The results of curve 2 were obtained with a solution to which Natulan was added immediately after termination of irradiation, whereas in the case of curves 3–5 increasing time intervals were inserted between the end of irradiation and the addition of Natulan. These curves demonstrate the very pronounced viscosity decrease which is obtained when Natulan is added to the DNA solution immediately after termination of irradiation, and that the effect of Natulan on the viscosity of irradiated DNA solutions decreases rapidly with increasing intervals between end of irradiation and addition of Natulan. DNA is apparently most susceptible to degradation by Natulan immediately after irradiation. It is assumed that this is due to peroxide products of the DNA formed in the course of irradiation. Most of these unstable products are known to decay within the first few hours after irradiation [6,19,30–35].

Fig. 3 is a further demonstration of the dependence of the combined effect of Natulan and irradiation on the time interval between irradiation and addition of the compound. Curve 1 shows the specific viscosity 240 hr after the addition of Natulan as a function of the time elapsed between end of irradiation and addition of the compound. This dependence of the reactivity of irradiated DNA solutions on their age is not observed with nitrogen mustard (curve 2). The action of this alkylating type of cytotoxic compound on DNA is apparently not influenced by the unstable products formed during the irradiation of DNA solutions.

3.3. Quantitative differences in the effects of Natulan and of nitrogen mustard on radiation-induced DNA degradation

Approximately the same decrease in viscosity of the 0.05% DNA solution is obtained with 0.0005 mol/l Natulan as with 0.0005 mol/l nitrogen mustard 240 hr after the addition of these compounds. However, when these compounds are given to freshly irradiated DNA solutions the additional effect due to irradiation on the viscosity decrease is much smaller in the case of nitrogen

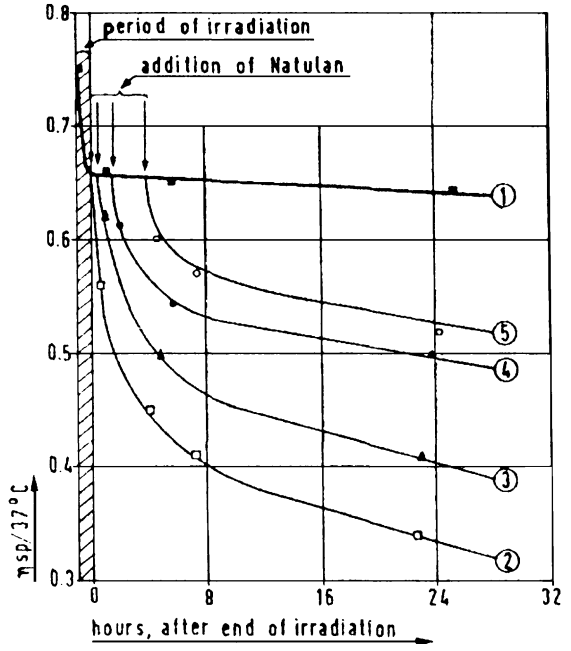

Fig. 2. Change in the specific viscosity of a 0.05% solution of DNA after irradiation with 11,000 rad and addition of 0.0005 mol/l *N*-isopropyl-*p*-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan) at various time intervals: (1) irradiated only, no addition of Natulan; (2) addition of Natulan immediately after termination of irradiation; (3) addition of Nalulan 30 min after termination of irradiation; (4) addition of Natulan 90 min after termination of irradiation; (5) addition of Natulan 235 min after termination of irradiation.

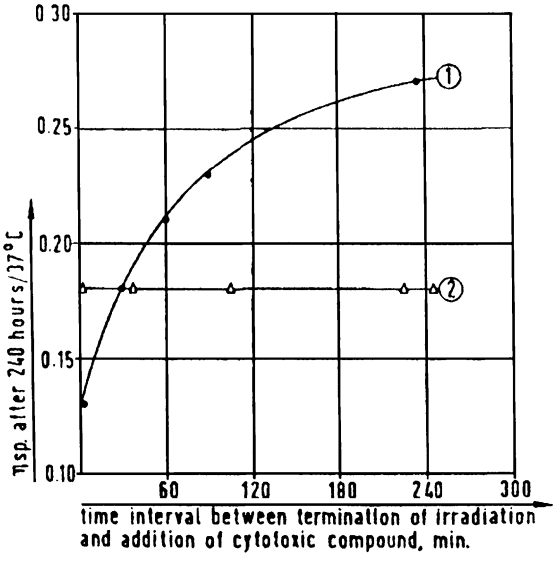

Fig. 3. Specific viscosity of a 0.05% solution of DNA irradiated with 11,000 rad and treated with the cytotoxic compounds Natulan or nitrogen mustard. Influence of the time interval between termination of irradiation and addition of the cytotoxic compounds on the specific viscosity (240 hr after irradiation); (1) 0.0005 mol/l *N*-isopropyl-*p*-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan®); (2) 0.0005 mol/l nitrogen mustard (methyl-bis(β-chloroethyl)amine hydrochloride).

Table 1
Specific viscosities of an 0.05% DNA solution 240 h after irradiation, after addition of the cytotoxic compounds, and after combination of irradiation and cytotoxic compounds

Irradiation only (11,000 rad)	Nitrogen mustard only (0.0005 mol/l)	Irradiation + nitrogen mustard	Natulan only (0.0005 mol/l)	Irradiation + Natulan®
0.61	0.27	0.21	0.28	0.08

mustard than in the case of Natulan. This is made evident by the experimental results given in Table 1.

3.4. Synergistic effect between varying doses of Natulan and a fixed radiation dose

In Fig. 4 sedimentation constants are presented for DNA degraded by different doses of Natulan with and without preceding irradiation. In the case of the irradiated samples Natulan was added immediately after irradiation. As has been demonstrated in the previous section the greatest effect with respect to viscosity decrease is obtained under these circumstances. After the addition of Natulan, all the samples were stored for 300 hr at 37 °C.

No further decrease of viscosity was observed after this time. The sedimentation constants given in Fig. 4 were obtained by extrapolation to the concentration limes zero.

In Fig. 5 the sedimentation constants of Fig. 4 were converted into "average molar concentrations" of DNA. For this calculation the relationship of Doty *et al.* [36] between average molar weight of degraded DNA and its sedimentation constant was used. Each fracture of the double chain of a DNA molecule yields a new DNA molecule. Therefore, the molarity of the solution

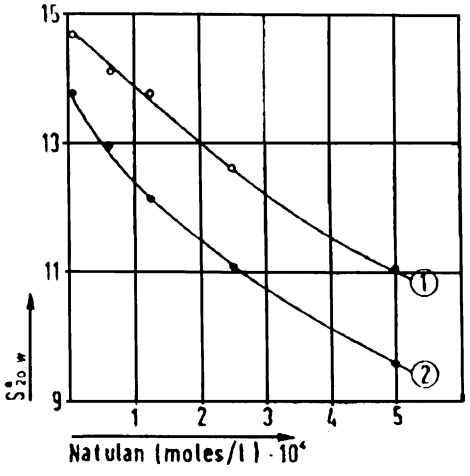

Fig. 4. Sedimentation constants of DNA after degradation with different doses of *N*-isopropyl-*p*-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan®)with and without preceding irradiation with 11,000 rad (addition of the compound immediately after termination of irradiation): (1) degradation by the compound alone; (2) degradation by the combined action of the compound and ionizing radiation (11,000 rad).

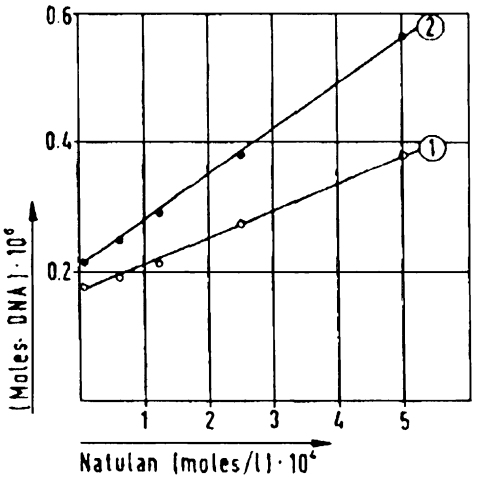

Fig. 5. Average molar concentration of DNA solutions with respect to DNA after treatment with different doses of *N*-isopropyl-*p*-(2-melhylhydrazino-methyl)benzamide hydrochloride (Natulan®) with and without preceding irradiation with 11,000 rad (addition of the compound immediately after termination of irradiation): (1) effect of Natulan alone; (2) effect of the combined action of Natulan® and of ionizing radiation (11,000 rad).

with respect to DNA increases proportionally to the number of double breaks. It follows that the increase in "average molar concentration" of the DNA is a measure for the number of double breaks that have occurred in DNA molecules. Fig. 5 represents the increase of the molarity of the DNA as a function of the concentration of Natulan added to the solution with and without preceding irradiation. From this graph it is seen that the "dose–effect curve" for the combined action of Natulan and irradiation is steeper than the corresponding curve for Natulan alone. The effect of irradiation is thus not just "additive" to the effect of Natulan, in which case two parallel curves should have been obtained. The effect of the two treatments is evidently greater than would be expected from a simple linear superposition.

3.5. Synergistic effect between varying doses of radiation and a fixed dose of Natulan

In Fig. 6 sedimentation constants are presented for DNA degraded by different doses of radiation with and without addition of a fixed dose of Natulan immediately after irradiation. The samples were stored for 300 hr at 37 °C before the analysis was done.

Fig. 7 gives the "average molar concentrations" of DNA calculated from the sedimentation constants of

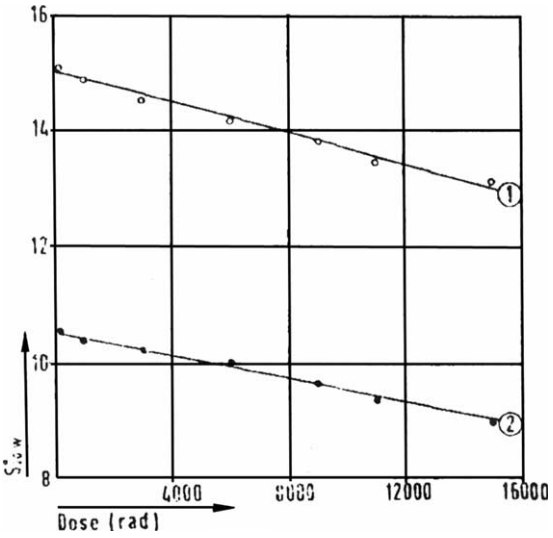

Fig. 6. Sedimentation constants of DNA after degradation by different doses of radiation with and without addition of *N*-isopropyl-(*p*-(2-metlylhydrazino-methyl)benzamide hydrochloride (Natulan®) (addition of the compound immediately after termination of irradiation): (1) irradiation alone; (2) combined action of radiation and of 0.0005 mol/l Natulan®.

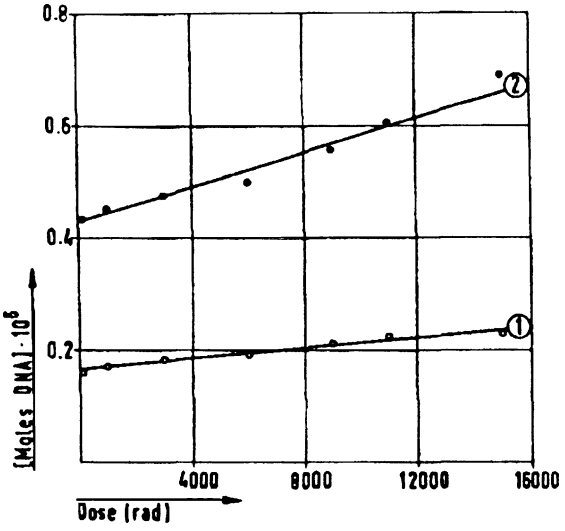

Fig. 7. Average molar concentration of DNA solutions with respect to DNA after degradation by different doses of radiation with and without addition of *N*-isopropyl-*p*-(2-methylhydrazino-methyl)benzamide hydrochloride (Natulan®) (addition of the compound immediately after termination of irradiation): (1) degradation by irradiation alone; (2) degradation by the combined action of radiation and 0.0005 mol/l Natulan®.

Fig. 6 according to the method described in the previous section. Again the curve for the combined action of irradiation and Natulan is steeper than the corresponding curve for irradiation alone, indicating that more DNA molecules are degraded by the combined treatment than would be expected from a simple summation of the effects of radiation and of the cytotoxic agent.

4. Discussion

The results presented here give further evidence that the combination of ionizing radiation and of Natulan® acts in a synergistic and not in an additive way on DNA. A mutual influence of the two treatments on each other must be assumed. No synergism was observed when irradiation was combined with either nitrogen mustard or hydrogen peroxide. As the synergistic effect decreases as the time interval between the end of irradiation and the addition of Natulan increases, it is assumed that unstable products formed during irradiation may be responsible for the synergism. Weiss *et al.* [30–32] and Butler *et al.* [6,18] have shown that organic peroxides are formed during irradiation of DNA solutions which show a marked post-irradiation decay. Butler *et al.* [6,7] suggests that this decay is even promoted by reducing substances (“reductive activation” [37]). The free radicals formed during this decay may act as initiators [37–39] of the autoxidation [2] of Natulan®. Earlier experimental evidence [4,5] supports the view that the autoxidation of Natulan is accelerated by reactions which supply free radicals, such as Fenton chain reactions. Radical scavengers, on the other hand, have a deleterious influence on the autoxidation of Natulan [3]. The higher rate of autoxidation will result in an increase of both the formation [2,3] and the activation [4,5] of hydrogen peroxide and thus enhance the degradation of DNA.

It had been claimed before that the action of Natulan on DNA *in vitro* shows marked similarities with the indirect effect of ionizing radiation [16], such as the intermediate formation of hydrogen peroxide [2,3] and of strongly oxidizing and reducing free radicals [2–5]. The results presented here give further support to this view. They are also of interest in connection with the mechanism of the autoxidation of Natulan for which a chain reaction involving free radicals is assumed [35,40]. Finally the experimental results may have some bearing on the interpretation of the “after effect” [6,7,15–29] of ionizing radiation. It has been claimed by several authors [16,30–32] that there may be some connection between the “after effect” of ionizing radiation and the post-irradiation decay of DNA-hydroperoxides. Our experimental results give further evidence to support this assumption.

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