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ADRIAMYCIN AND DAUNOMYCIN INDUCE INTERSTRAND DNA CROSSLINKS IN HeLa S3 CELLS

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SUMMARY: By using a new mild procedure for detecting DNA crosslinks it has been shown that adriamycin and daunomycin are able to form interstrand DNA crosslinks in HeLa cells. This effect seems to be preceded by transformation of the parent antibiotics in the cell to active forms. In addition, interstrand DNA crosslinks formed by adriamycin and daunomycin were found to be temperature- and alkali-labile.

The antitumor and cytotoxic properties of adriamycin and daunomycin have been associated with intercalation of these antibiotics to DNA and subsequent blocking of nucleic acid synthesis[1-4], though importance of intercalative binding to DNA, for biological activity of these antibiotics has not been unequivocally established. Resent studies have shown that intercalation is not responsible for the high cytotoxic and antitumor properties of some acridine derivatives which otherwise belong to a group of classical DNA-intercalating agents[5-7]. Furthermore 1-nitroacridine derivatives were shown to form crosslinks in cellular DNA and that this effect played an important role in their mode of action[8,9]. These findings suggest that some other highly cytotoxic and antitumor compounds believed to act by intercalation to DNA, could form interstrand DNA crosslinks.

Abbreviations: RF, renaturable fraction of DNA, ΔRF increase in renaturable fraction of DNA.

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MATERIALS AND METHODS: HeLa S3 cells (0.7×10^6) were plated in 20 cm^2 Leighton tube containing 10 ml MEM with 5% foetal calf serum and cultivated for 48 hrs in 5% CO_2, at 37°C. Tissue culture supplies were Gibco-Biocult, Scotland. The medium was replaced with a fresh one with 1μCi/ml [^{3}H-methyl] thymidine (23 Ci/mmol, UVVR, Czechoslovakia) for further 16 hrs. Then the cells (5×10^5/tube) were treated for 3 hrs with drugs at concentrations specified in the tables (duplicate tubes for each concentration). After incubation, the cells were trypsinized and immediately suspended in a medium containing serum, to terminate the action of trypsin, and were combined with the the cells recovered from the incubation medium. The cells were washed with cold saline and delipidated by subsequent washings with 96% ethanol, ethyl ether and 96% ethanol. The cells were suspended in ethanol (0.3 ml) and lysed by addition of 3.5 ml 6 M sodium thiocyanate. After 3 hrs at room temperature sodium thiocyanate was removed by thin layer dialysis[18] (3 x 0.5 hr at 0°C) against 2 mM Tris-HCl, pH 7.0 buffer containing 1 mM EDTA (4 liters per 4 samples). The samples were then digested with proteinase K (200 μg/ml, Merck, West Germany) in 10 mM Tris-HCl, pH 8.0 buffer containing 0.2% SDS, 10 mM EDTA and 10 mM NaCl, for 2 hrs at 40°C. Unless specified otherwise the DNA in the digests was denatured by thin layer dialysis against formamide (300 ml/sample, 3 hrs, 40°C) and then renatured by dilution of the sample (2:1) with 0.02 M acetate buffer, pH 6.0, and thin layer dialysis against 0.002M acetate buffer, pH 5.5 (3 x 0.5 hr, 1 liter/sample). After addition of nuclease S1 buffer (final concentration:3.9 mM acetate, 0.1M NaCl, 5 mM Zn^{+2}, pH 4.4) the samples were sonicated (10 sec, 100 W) and heated 15 min at 55°C. Six aliquots of each sample were taken. Half of them was next incubated for 1 hr. at 40°C with 100 U/1.5 ml of Sigma, USA or Boehringer Mannheim, West Germany nuclease S1, while the other half was incubated in the same way without nuclease S1 (for total radioactivity of the sample). Then the tubes were chilled on ice and DNA was precipitated by addition of cold TCA up to 7.5%. After 30 min at 0 - 4°C the precipitates were collected onto nitrocellulose filters. After washing with 10% TCA, methanol-chloroform (1:1), the filters were dried and their radioactivity was determined in a standard toluene scintillation fluid. Renaturable fraction of DNA (RF) was calculated as radioactivity of nuclease S1 digested sample, divided by total radioactivity of nondigested sample, expressed in per cent. Renaturable fraction of DNA for control cells (average from 40 determinations) amounted to 15.5±0.8%. Increase of renaturable fraction of DNA (ΔRF) was calculated by subtracting RF for control from the values of RF for drug - treated samples.

RESULTS AND DISCUSSION: The ability of adriamycin and daunomycin to form interstrand DNA crosslinks in tumor cells was studied by a new, mild procedure based on increased renaturability of crosslinked DNA, where nuclease S1[10,11] was used for estimation of the renaturable fraction of DNA.

Adriamycin and daunomycin induce breaks in DNA[4,12], which could markedly decrease the sensivity of DNA crosslinks detection

TABLE 1. Increase of renaturable fraction of DNA (ΔRF) of HeLa S3 cells incubated with adriamycin, daunomycin and mitomycin C.

compound	concentration	Δ RF ± SEM	n*
adriamycin	10 μM	11.0 ± 2.0%	8
	50 μM	24.8 ± 2.5%	25
	150 μM	41.7 ± 5.0%	8
daunomycin	10 μM	15.3 ± 1.3%	4
	20 μM	16.2 ± 1.4%	4
	50 μM	20.8 ± 3.3%	8
Ledakrin	2.5 μM	22.0 ± 4.0%	4
mitomycin C	20 μM	10.9 ± 0.7%	4

n* - number of experiments.

based on increased renaturability of crosslinked DNA. Therefore, these studies required a highly sensitive method. The employed procedure was carried out in cell lysates, without isolation of DNA, in order to minimize further DNA degradation and thereby to increase the sensivity of DNA crosslink detection. Cells were lysed with 6M sodium thiocyanate[13], and the cell lysates were digested with proteinase K to remove cellular protein that prevents the adequate denaturation of DNA. Furthermore, denaturation of DNA carried out in formamide provided milder conditions than thermal and alkaline denaturation, increasing therefore the chances for the detection of unstable DNA crosslinks.

Employing this procesure, it was found that adriamycin and daunomycin considerable increase, in a dose dependent manner, the renaturable fraction of DNA (RF) from HeLa S3 cells incubated 3 hours with the antibiotics (Table 1). Ledakrin, and mitomycin C, already known as DNA-crosslinking agents[8,9] were used as positive controls. The latter also produced an increase

TABLE 2. Increase of renaturable fraction (ΔRF) of DNA of HeLa S3 cells lysates exposed to adriamycin and daunomycin.

compound	equivalent to conc.*	ΔRF ± SEM	n
adriamycin	50 μM	-0.5 ± 2.0%	12
	150 μM	-1.4 ± 8.5%	3
daunomycin	50 μM	-3.7 ± 2.5%	6

The drugs were added to the lysates of HeLa S3 cells ($5x10^6$ cell/sample) after removal of sodium thiocyanate and adjusting pH to 7.0 and incubated 1 hr at 4°C in dark. Then the unbounded antibiotics were removed by thin layer dialysis to 2 mM Tris-HCl buffer, pH 7.0 containing 1 mM EDTA. The samples were further processed as described in Materials and Methods.

* The concentrations specified in this table would be obtained if the amount of the drug actually added to the lysates would be given to the cells in the experiments shown in Table 1.

n - number of experiments.

of the renaturable fraction of DNA (ΔRF) in HeLa S3 cells (Table 1).

It is interesting to note that anthracycline antibiotics that are around 10 times less cytotoxic than ledakrin, produce an increase of the renaturable fraction of DNA that is comparable to that for ledakrin but at 10 times lower concentration.

One could consider the increased renaturable fraction of DNA in the cells incubated with adriamycin to be a result of intercalation of these antibiotics to DNA. To rule out this possibility I assayed the effect of the antibiotics added to the lysates from the HeLa S3 cells (after removal of sodium thiocyanate). The amount of anthracyclines with regard to the number of cells prior to lysis was the same as in previous experiments. Except for one additional thin layer dialysis, to remove the unbound antibiotics, the further procedure was the same as previously. As shown on Table 2, the addition of the drugs to the lysate caused no increase of the renaturable fraction of DNA. This

means that intercalative complexes of anthracycline antibiotics formed with DNA in HeLa S3 lysates, were disrupted by formamide during denaturation processes. Hence, the observed increase of renaturable fraction of DNA from antibiotic treated cells, is not due to the intercalation of anthracycline to DNA and could be interpreted as the formation of interstrand DNA crosslinks by adriamycin and daunomycin. In addition, these data show that adriamycin and daunomycin given to the whole cell undergo metabolic transformation that is not possible under the conditions of the lysates. This metabolic transformation changes the parent antibiotics into an active form, able to crosslinks DNA. In this respect, anthracycline antibiotics thus resemble ledakrin, mitomycin C and cyclophosphamide, in that they require to be activated to be able to form interstrand DNA crosslinks.

Thermal denaturation of DNA in lysates from HeLa S3 cells incubated with adriamycin and daunomycin, in contrast to milder formamide denaturation, prevents increase of the renaturable fraction of DNA. The same was observed when crosslinked DNA after formamide denaturation and renaturation, was subjected to an additional thermal denaturation prior to final renaturation (Table 3).

In the case of ledakrin, shown previously to form temperature-stabile crosslinks[8,9], the increase in the renaturable fraction of DNA after denaturation, is similar to that after formamide denaturation (Tables 1 and 2).

Alkaline denaturation of DNA in the procedure with cell lysates could not be used, because it results in a too high renaturable fraction of control DNA, as shown by preliminary experiments. This arises in all probability from the very high molecular weight of cellular DNA. Introduction of additional breaks to DNA by X-ray irradiation[14,15], could overcome this

TABLE 3. The influence of thermal and alkaline methods of DNA denaturation on the increase of renaturable fraction of DNA (ΔRF) of HeLa S3 cells incubated with anthracycline antibiotics and ledakrin.

compound	conc.	denaturation of DNA	Δ RF ± SEM	n*
adriamycin	50 μM	thermal	-0.5 ± 2.0%	4
daunomycin	50 μM	thermal	0.3 ± 0.5%	3
Ledakrin	2.5 μM	thermal	25.3 ± 5.3%	4
adriamycin	50 μM	formamide followed by thermal	0.3 ± 0.2%	3
adriamycin	50 μM	formamide followed by alkaline	-1.4 ± 1.5%	3

The procedure was essentially the same as in Table 1, except for denaturation/renaturation of DNA, that was carried out as specified in the table. For the thermal denaturation the pH was adjusted to 7.0 and the samples were heated at 100°C for 15 min. followed by rapid cooling. For alkaline denaturation 1N NaOH was added to give 0.03 N final concentration. After 15 min at room temperature (in the dark) the samples were neutralized with acetic acid.

*n - number of experiments.

difficulty, but also would decrease the sensivity of detecting DNA crosslinks. On the other hand additional alkaline denaturation of DNA, after formamide denaturation and renaturation, gave a low renaturing fraction for the control DNA and no increase (ΔRF=0) for adriamycin (Table 3) and daunomycin (data not shown). Hence the interstand DNA crosslinks formed by adriamycin and daunomycin are sensitive to alkali.

Though this conclusion should be confirmed in a more direct way, it explains why interstrand DNA crosslinking by anthracycline antibiotics was not detected by alkaline elution method, employed for studies of adriamycin and daunomycin interaction with cellular DNA by several authors[16,17].

Reported in this paper ability of adriamycin and daunomycin to form interstrand crosslinks in DNA of cultured HeLa cells, was found using only one technique, and one method of DNA

denaturation (by formamide). Confirmation of this finding in another way seems very important but may not be easy to achieve because of the observed instability of the anthracycline formed crosslinks.

The formation of interstrand DNA crosslinks by adriamycin and daunomycin might be essential for their biological activity, though this remains to be established in the further studies.

On the other hand, the results reported in this paper, as well as our previous studies with 1-nitroacridines[6,8,9], support the suggestion that some other highly cytotoxic and antitumor intercalating agents could also form interstrand DNA crosslinks.

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