Inhibition of DNA and RNA Synthesis by Daunorubicin in Sensitive and Resistant Ehrlich Ascites Tumor Cells in Vitro

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SUMMARY

The effect of daunorubicin (DNR) on nucleic acid synthesis was investigated in vitro in cells from an Ehrlich ascites tumor that was sensitive in vivo to DNR and in cells from a subline of the tumor in which resistance to DNR had been developed in vivo.

DNR inhibited DNA and RNA synthesis in sensitive as well as in resistant cells, as determined from the incorporation of three different radioactive precursors. In order to give the same degree of inhibition of DNA and RNA synthesis in resistant cells as in sensitive cells, the DNR concentrations required were approximately 5 times greater.

When the synthesis of different RNA species in sensitive cells was analyzed, nucleoplasmic heterodisperse RNA was found to be the least DNR sensitive, while tRNA, 5 S RNA, and two small-molecular-weight RNA components had a medium sensitivity to DNR. Labeling of rRNA and of the 45 S and 32 S nucleolar RNA was found to be inhibited most and to the same extent, indicating that it is the synthesis of 45 S RNA that is inhibited and not the processing of 45 S RNA to rRNA. The preferential inhibition of rRNA was less pronounced in resistant cells than in sensitive cells, thus indicating that the sensitivity of RNA synthesis was changed more during development of resistance than was the sensitivity of synthesis of other RNA components.

The cellular uptake of DNR was less in resistant than in sensitive cells, but the difference was too small to explain the differences in inhibition of the nucleic acid synthesis.

INTRODUCTION

DNR is an antibiotic that plays an important role in the treatment of acute leukemias (1, 5). It is an anthracycline and has been shown to inhibit cellular synthesis of both DNA and RNA (7, 19, 20), probably by binding to DNA (3, 6, 25).

In previous studies, resistance to DNR was developed in vivo in an Ehrlich ascites tumor by daily treatment with DNR in 17 weekly passages. Growth of the resistant line was not inhibited by treatment for 5 days with a 10% lethal dose of the drug, compared to an inhibition of the sensitive tumor by the same dose to 8% of the growth of the control cells (4).

MATERIALS AND METHODS

Tumor Cells. The near-tetraploid Ehrlich ascites tumor EHR 2 and the corresponding DNR-resistant tumor EHR 2/DNR+ (4) were used in all experiments. Ascites fluid was removed for in vitro experiments 6 to 8 days after inoculation of the tumor. No DNR was given to the DNR-resistant tumor in the last passage before in vitro experiments. Prelabeling of cells for 48 to 72 hr with Na2H32PO4 was done by i.p. injection of the isotope dissolved in 0.9% NaCl solution.

Drug and Isotopes. DNR as daunomycin hydrochloride (Farmitalia Co., Milan, Italy) was kindly supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, NIH, Bethesda, Md. The drug was dissolved in 0.9% NaCl solution immediately before use. The concentration was controlled spectrophotometrically before each experiment.

Thymidine-2-14C, uridine-5-3H, and adenine-2-3H were obtained from the Radiochemical Centre, Amersham, England; cytidine-3H(G) was obtained from NEN Chemicals, Frankfurt am Main, Germany; and Na2H32PO4 was obtained from The Danish Atomic Energy Commission, Risø, Denmark.

Incubation. Ascites tumor cells were washed 2 to 4 times at 2–4° in Earle’s solution (9) by centrifugation at 900 X g until free from erythrocytes. After resuspension, the volume % of cells was determined by centrifugation of a sample at 500 X g for 5 min in a hematocrit centrifuge, and the suspension was diluted with Earle’s solution, supplemented with 10% calf serum, and equilibrated with a gas mixture of 95% atmospheric air and 5% CO2 to give a final density of 10 µ tumor cells/ml, which was used throughout the study. Volumes varying from 10 to 80 ml were incubated in Erlenmeyer flasks at 37° in a water-thermostated shaking incubator in an atmosphere composed of 95% atmospheric air and 5% CO2. DNR and radioactive precursors were added as indicated in each experiment. The pH remained between 7.0 and 7.4 during incubation for 3 hr, and a viability test with nigrosin (15) on the control cells always showed more than 95% viable cells both before and after incubation. Incubation was stopped by placing the flasks in ice water.

Specific Activity of DNA and RNA. The technique used has

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1 This investigation was supported by the Danish Medical Research Council and the Novo Foundation.
2 The abbreviations used are: DNR, daunorubicin; SDS, sodium dodecyl sulfate; MAK, methylated albumin-Kieselguhr.

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been described previously (10). After treatment of the cells with ice-cold perchloric acid, the pellet was washed with perchloric acid, alcohol, and alcohol : ether and then digested in 0.5 N KOH at 37° for 16 to 18 hr. The solution was acidified and centrifuged to give the ribonucleotides in the supernatant fluid. DNA in the pellet was washed and digested in trichloroacetic acid at 90° for 30 min to give the deoxyribonucleotides. Ribonucleotides and deoxyribonucleotides were determined by sugar reactions for ribose and deoxyribose, respectively, and radioactivity was measured in a liquid scintillation spectrometer (12).

RNA Extraction from Whole Cells. RNA was extracted with phenol and potassium phosphate buffer (pH 7.2) at 0° as previously described (23). RNA in the aqueous phase was precipitated with alcohol and termed “cytoplasmic” RNA. The interphase was extracted for 15 min at 70° with 5% SDS and phenol in an acetate buffer (pH 5.1). The extracted RNA was termed “nRNA-I.” RNA remaining in the last interphase was released as nucleotides after digestion in 0.5 N KOH and was termed “nRNA-II.” The specific activity of the 3 RNA fractions was determined by a procedure identical to that described above.

Nucleolar and Nucleoplasmic RNA. Nucleoli were prepared by a method described by Willems et al. (29) for HeLa cells with some modifications. After swelling in 10 ml hypotonic buffer (0.01 M NaCl solution, 0.01 M Tris-HCl, and 0.0015 M MgCl₂, pH 7.4), whole cells with a packed volume of 100 μl were homogenized with a close-fitting ball homogenizer. After centrifugation, the pellet, consisting mainly of nuclei, was washed and resuspended in cold hypotonic buffer, which was made 1% with the detergent Tween 40 and 0.5% with sodium deoxycholate and shaken for 3 sec on a Whirlimixer. After being washed with hypotonic buffer, the pellet was resuspended in 1 ml hypertonic buffer (0.8 M NaCl solution, 0.05 M MgCl₂, and 0.01 M Tris-HCl, pH 7.4) and digested with 50 μg DNase (Worthington, electrophoretically purified) for 75 sec at 37°, under vigorous stirring.

The digested nuclei were layered on 30 ml of a 15 to 30% (w/v) gradient of sucrose in the hypertonic buffer and centrifuged at 30,000 X g for 15 min in an SW 25.1 rotor in a Spinco ultracentrifuge. The pellet was termed “nucleoli” and the supernatant fluid was termed “nucleoplasm.” The nucleoplasm was decanted and precipitated by addition of 2 volumes of 95% ethanol at −18°.

RNA from nucleoli and from nucleoplasmic precipitate was extracted with hot phenol : chloroform : SDS at 55° (24).

For determining inhibition of uridine incorporation into nucleolar and nucleoplasmic RNA, 2 equal batches of cell suspensions were labeled with uridine-5³H with and without DNR and then mixed with equal volumes of cell suspensions that had been labeled with Na₂H³²PO₄ without DNR added. The ratio ³H counts : ³²P counts in isolated RNA fractions from cells labeled with uridine-5³H in the presence of DNR, as a percentage of the corresponding ratio in control cells, was taken as a measure of the degree of inhibition by DNR.

Fractionation of RNA. Fractionation of cytoplasmic RNA in tRNA + 5 S RNA and rRNA by MAK column chromatography was performed as described previously (13). Fractionation of RNA by polyacrylamide gel electrophoresis was, with some modifications, performed as described by Loening (16) and by Weinberg et al. (26).

Gels with 7.4% acrylamide (Serva Chemicals, Heidelberg, Germany) and 0.19% bisacrylamide (Serva) were cast in tubes 6 mm in diameter with buffer (0.017 M sodium acetate, 0.04 M Tris, and 0.002 M EDTA, pH 7.8), 0.24% N,N,N',N'-tetramethylethylenediamine, and 0.24% ammonium persulfate. Electrophoresis was performed with the same buffer. Gels with 2.5 or 3.0% acrylamide and 0.17% bisacrylamide were cast in tubes 6 mm in diameter with buffer (0.05 M NaH₂PO₄, 0.04 M Tris, 0.002 M EDTA, and 10% glycerol, pH 7.4), 0.12% N,N,N',N'-tetramethylethylenediamine, and 0.10% ammonium persulfate. Electrophoresis was performed with the same buffer made 0.5% with respect to SDS.

Electrophoresis was performed at room temperature. Voltage gradients up to 5 V/cm were used. After electrophoresis, 7.4% gels were cut in 2.5-mm slices on a fakibed of razor blades, while 2.5 and 3.0% gels were frozen with Dry Ice and sliced in 2-mm slices with a Mickle gel slicer.

Gel slices were digested with Nuclear Chicago solubilizer and counted in toluene : dioxane : ethanol scintillation fluid in a scintillation spectrometer (11).

Cellular Uptake of Nucleic Acid Precursors. Cells were incubated for 30 min with DNR as described in Table 1, and the radioactive precursor was then added. After incubation for a 30 min longer, the cells were centrifuged, and the amount of radioactive precursor remaining in the medium was determined. The initial value of precursor in the medium was determined in a separate experiment in which the radioactive precursor was added to samples of the medium after removal of the cells. Three cell batches were used for each determination, and the mean was calculated.

Measurement of DNR in Medium and Cells. After incubation with DNR, the cell suspensions were cooled to 0–4° and the cells were pelleted by centrifugation at 900 X g for 10 min. The cells were washed twice with 10 ml 0.9% NaCl solutions and extracted once with SDS : phenol : chloroform at 55° as described above for extraction of RNA.

DNR concentrations in medium, wash fluid, and cell extract were determined spectrophotometrically at 480 nm. The total amount of DNR recovered in medium, wash fluid, and cells was more than 90% in all experiments. The cellular uptake of DNR was determined from the amounts extracted from the cells but was also calculated from the difference between the amount of drug added and the amount found in medium and wash fluid. The mean values of these 2 figures were plotted versus time and are shown in Chart 5.

RESULTS

Inhibition of Labeling of DNA and RNA. As shown in Charts 1 and 2, labeling of DNA and RNA with thymidine-¹⁴C and uridine-5³H, respectively, was decreased by DNR in both sensitive and resistant cells. The inhibitory effect of DNR is exerted prior to 5 min, when the 1st determinations have been made.
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Chart 1. Inhibitory effect of DNR on DNA synthesis in sensitive and resistant Ehrlich ascites tumor cells. One % suspensions were preincubated with DNR for 30 min followed by incubation with thymidine-\(^{14}\)C (0.1 \(\mu\)Ci/ml, 20 \(\mu\)Ci/\(\mu\)mole) for 30 min. The specific activity of DNA at each DNR concentration was expressed as percentage of the mean of the specific activities in 3 control flasks in the absence of DNR. Inset, the incorporation with time under identical incubation conditions with various DNR concentrations as indicated. In these experiments, both DNR and tracer were added at Time 0.

Chart 2. Inhibitory effect of DNR on RNA synthesis in sensitive and resistant Ehrlich ascites tumor cells. Conditions as indicated in Chart 1, except that labeling was performed with uridine-\(^{3}\)H (3 \(\mu\)Ci/ml, 30 \(\mu\)Ci/\(\mu\)mole). Inset, time lapse of the incorporation under identical incubation conditions with various DNR concentrations as indicated. In these experiments, both DNR and tracer were added at Time 0.

The dose-response curves show that for both cell types a partial inhibition of labeling of RNA with uridine-\(^{3}\)H was obtained with low DNR concentrations that did not inhibit labeling of DNA with thymidine-\(^{14}\)C. The dose-response curves for RNA were less steep than those obtained for DNA, and complete inhibition of labeling of RNA was first seen at doses that also inhibited labeling of DNA completely.

DNR concentrations about 5 times higher for resistant cells...
Inhibitory effect of DNR on incorporation of various precursors into DNA and RNA in sensitive and resistant Ehrlich ascites tumor cells

Table 1

| Table 1 | Inhibitory effect of DNR on incorporation of various precursors into DNA and RNA in sensitive and resistant Ehrlich ascites tumor cells |

Table 1 shows the inhibitory effect of DNR on the incorporation of thymidine (2 concentrations), cytidine, and adenine into DNA and the inhibition of incorporation of uridine (2 concentrations), cytidine, and adenine into RNA. In all cases, only insignificant differences in the degree of inhibition were obtained with different precursors (a t test showed p > 0.025 in all cases).

In identical experiments, the cellular uptake of the precursors was determined. The uptake of uridine, cytidine, and adenine into sensitive and resistant cells was not affected by the presence of DNR in the concentrations used. The uptake of thymidine into both sensitive and resistant cells was inhibited by DNR to approximately 70% of the controls. In the case of thymidine, a relatively larger amount of radioactive incorporation was found in the acid-soluble fraction than for sensitive cells. Radiolabeled DNA was separated into tRNA, 5 S RNA, Component D, Component C, and the rRNA in the top slice of the gel as described previously (14). Components D and C are small-molecular-weight RNA components corresponding to those found in HeLa cells (27) and Novikoff hepatoma cells (17). The rRNA was separated in 18 S and 28 S RNA by electrophoresis on 3% gels (Chart 3).

When experiments were performed with resistant cells instead of sensitive cells, and cytoplasmic RNA was analyzed by electrophoresis on 7.4 and 3% gels, the labeling profiles and the specific activity of each RNA component in the control cells were similar to those obtained with sensitive cells. The inhibitory effect of DNR on the labeling of the different cytoplasmic RNA components that were isolated by gel electrophoresis was determined from the $^3H:32P$ ratios and is shown in Table 2. In sensitive cells, labeling of 18 S and 28 S RNA was most decreased, and to the same extent. In resistant cells, labeling of 18 S and 28 S was also decreased more than that of other cytoplasmic RNA components; but as was the case in the experiments performed with fractionation by MAK chromatography, the preferential inhibition of rRNA was less pronounced in resistant than in sensitive cells.

Chart 4 shows the labeling profiles after gel electrophoresis of nucleolar RNA from sensitive cells incubated with Na$_2^{32}$PO$_4$ and uridine-$^3$H, with or without DNR. Two peaks were termed 45 S and 32 S RNA, according to the nomenclature for nucleolar RNA species identified as rRNA precursors in HeLa cells (28), and the labeling of both of these RNA species was decreased by DNR. Peaks with similar mobility as in sensitive control cells and with similar ratios between $^3H$ counts and $^32P$ counts were found after electrophoresis of nucleolar RNA prepared in the same way from resistant cells. As shown in Table 2, the labeling of 45 S and 32 S was inhibited by DNR in both sensitive and resistant cells, but the sensitivity of resistant cells was more than 5 times less than that of sensitive cells. The degree of inhibition is similar for 45 S and 32 S RNA and is equal to the degree of inhibition of labeling of 18 S and 28 S rRNA in the same cell type.
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On the labeling profile of nucleoplasmic RNA (not shown), there were small peaks at 32 S and 28 S plus a considerable amount of heterodisperse RNA. $^{32}P$ and $^3H$ counts in heterodisperse RNA migrating between the 28 S and 32 S RNA peaks and slower than the 32 S peak were totaled, and the degree of inhibition was calculated from these figures. The results given in Table 2 show that, in both sensitive and resistant cells, labeling of nucleoplasmic heterodisperse RNA is only slightly inhibited by DNR at the dosages used.

**Cellular Uptake of DNR.** For an investigation of whether the differences in inhibition of labeling of nucleic acids in sensitive and resistant cells could be attributed to differences in the cellular uptake of the drug, the concentration of DNR in the medium and the cells was determined with time and the uptake calculated, as shown in Chart 5.

In sensitive cells the uptake is rapid. With initial DNR concentrations of 2 and 10 μg/ml, the uptake has reached a plateau within 15 min. With 50 μg/ml, the curve levels off after 30 min, but the uptake still continues at a very decreased rate. The drug is strongly concentrated in the sensitive cells, and after 15 to 30 min, more than 75% of the drug is found in the cells in the 1% cell suspension. This means that the average concentration in the cells is more than 300 times higher than the concentration in the surrounding medium.

In the resistant cells, the DNR uptake is slower than in sensitive cells and attains a lower level in the period investigated. When compared with the uptake of DNR in sensitive cells, only between 40 and 75% of this amount is taken up in resistant cells after 30 min of incubation.

**DISCUSSION**

DNR is shown to inhibit the incorporation of several different radioactive precursors into DNA and RNA in sensitive and resistant Ehrlich ascites tumor cells. The inhibitory effect of DNR seems to be exerted on the polymerization of DNA and RNA, since incorporation of each of the precursors, adenine, cytidine, and thymidine, into RNA was inhibited to about 70% of the controls, but the inhibitory effect of DNR on the incorporation of nucleic acids in sensitive and resistant cells could be attributed to differences in the cellular uptake of the drug, the concentration of DNR in the medium and the cells was determined with time and the uptake calculated, as shown in Chart 5.

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DNR is shown to inhibit the incorporation of several different radioactive precursors into DNA and RNA in sensitive and resistant Ehrlich ascites tumor cells. The inhibitory effect of DNR seems to be exerted on the polymerization of DNA and RNA, since incorporation of each of the precursors, adenine, cytidine, and thymidine, into DNA was inhibited to the same extent, and this was also the case for adenine, cytidine, and uridine into RNA. These findings make it unlikely that DNR exerts its effect by inhibiting an enzyme in nucleotide metabolism but it could still be a general inhibitor of the cellular uptake of the precursors. The uptake of radioactive adenine, cytidine, and uridine into sensitive and resistant cells, however, is not affected by the concentrations of DNR used in the inhibition experiments. The total uptake of thymidine is inhibited to about 70% of the controls, but the radioactivity present in the acid-soluble pool is the same in control and DNR-treated cells. It is therefore concluded that the inhibitory effect of DNR on the incorporation of radioactive precursors into DNA and RNA is due to an inhibition of the polymerization of the nucleotides. It has been shown previously that DNR inhibited labeling of DNA and RNA in vivo and in cultured cells (6, 7, 19–21). It was supposed that the effect was due to binding of DNR to DNA (3), possibly by intercalation between adjacent base pairs in helical DNA (25). The present results are consistent with this.

The incorporation of uridine-$^3H$ into different RNA components was inhibited to a different extent by DNR. The
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Control

18S RNA

20S RNA

2 μg/ml

18S RNA

10^6 counts per min

Migration in gel (cm)

Chart 3. The inhibitory effect of DNR on the labeling of rRNA in sensitive Ehrlich cells incubated with and without DNR. Ascites tumor cells were labeled in vivo by i.p. injection of Na₂H³PO₄ (50 μCi/mouse) about 72 hr before cells were removed. One % cell suspensions were precultured with and without DNR for 15 min, followed by incubation for 180 min with uridine-5⁻³H (6 μCi/ml, 60 μCi/μmole). "Cytoplasmic" RNA was extracted, and electrophoresis on 3.0% polyacrylamide gels was performed with 2200 V X min/cm. The radioactivity in gel slices 2 mm thick was counted in a liquid scintillation spectrometer.

synthesis of heterodisperse, high-molecular-weight, nucleoplasmic RNA has relatively little inhibition, whereas the synthesis of tRNA, 5 S RNA, and 2 small-molecular-weight RNA components (Components D and C) shows medium sensitivity to DNR. In contrast, DNR inhibits the synthesis of 18 S and 28 S rRNA and the nuclear 45 S and 32 S precursors strongly and to the same extent, thus indicating that it is the synthesis of 45 S RNA that is inhibited and not the processing of 45 S RNA to rRNA. This conclusion supports a recent finding that in L1210 cells DNR does not inhibit the processing of 45 S RNA to rRNA, as is the case with other supposed intercalating agents (22).

The preferential inhibition of rRNA by DNR is in agreement with findings by Rusconi and Calendi (19) that, in HeLa cells, labeling with adenine-8⁻¹⁴C was totally inhibited in rRNA by DNR in doses that permitted some labeling of 4 S RNA. The preferential inhibition of labeling of nucleolar RNA is in agreement with electron microscopic studies by Dorigotti (8), who found pronounced nucleolar alterations as a primary effect of DNR in HeLa cells, and with radioautographic studies by DiMarco et al. (7) showing preferential inhibition of nucleolar uridine-³H labeling by DNR in HeLa cells.

The inhibition of labeling of DNA and RNA by DNR was different in sensitive and resistant cells, the main difference being that, in order to give the same degree of inhibition in resistant as in sensitive cells, the concentrations required are 5 times as high. These experiments in vitro indicate that the tumor is not totally resistant to DNR. It has not been possible to obtain an inhibition of the resistant tumor in vivo. A dose of DNR equal to a 10% lethal dose did not inhibit growth of the resistant tumor, compared to an inhibition of the growth of the sensitive tumor to about 10% of the control (4). The effect of higher doses of DNR could not be tested because of the toxic effect on the animals. However, it is possible that mechanisms of resistance are active in vivo that cannot be detected in the present investigation of explants of the tumor.

As in sensitive cells, the synthesis of rRNA in resistant cells was more inhibited by DNR than was the synthesis of other components, but the preferential inhibition of rRNA was less pronounced in the resistant cells. This may indicate that the sensitivity to inhibition of synthesis of rRNA was more altered during developments of resistance to DNR than that of other RNA components.

One process involved in the development of resistance is the
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Chart 4. The inhibitory effect of DNR on the synthesis of nucleolar RNA in sensitive cells. A cell suspension was preincubated with DNR for 15 min, labeled for 90 min with uridine-5-³H (18 μCi/ml, 180 μCi/μmole), and pooled with equal volumes of a similar suspension labeled for 90 min with NaH₂¹⁹PO₄ (100 μCi/ml) in the absence of DNR. Control cells were treated in same way, except for omission of DNR. RNA was extracted from the nucleoli with phenol : chloroform : SDS at 55° and subjected to electrophoresis on 2.5% polyacrylamide gels with 3800 V x min/cm.

Chart 5. Cellular uptake of DNR in sensitive and resistant Ehrlich ascites tumor cells. One % cell suspensions were incubated at 37°, and DNR was added at Time 0 to give the concentrations indicated. The amount of DNR remaining in the medium and in 2 batches of wash fluid and the amount of DNR extracted from the cells with phenol : chloroform : SDS were determined spectrophotometrically; the amount of drug taken up was calculated as described in "Materials and Methods." When the initial DNR concentration was 2 μg/ml, the data were based only on the DNR extracted from the cells.

cellular uptake of DNR. Depending on the initial concentration of DNR, the uptake of DNR in resistant cells determined after 30-min incubation was between 40 and 75% of that in sensitive cells. The present experiments do not establish whether the difference is due to altered membrane transport of DNR, altered binding capacity for DNR of some cell components, or both. Biedler and Riehm (2, 18) developed resistance to DNR in Chinese hamster cells in vitro and found a markedly decreased uptake in resistant cells in autoradiographic studies with tritiated DNR. Thus, decreased uptake may be a general phenomenon in acquired resistance to DNR.

The decreased uptake of DNR can partially explain why a smaller inhibition of nucleic acid synthesis is seen in resistant than in sensitive tumor cells. However, the finding that the sensitivity of different RNA species is changed to varying degrees indicates that the decreased uptake of DNR is not the only explanation. This assumption is supported by the finding that, when DNR is added to cell suspensions to give a concentration of 2 μg/ml with sensitive cells and 10 μg/ml with resistant cells, about the same degree of inhibition of labeling of total DNA and RNA is obtained in the 2 cell types, despite the fact that with these concentrations about twice as much DNR is taken up in resistant as in sensitive cells. The mechanism of resistance to DNR thus appears to be composed of several factors. Decreased uptake of DNR is a factor; other
possible factors may be an altered sensitivity of the RNA- and DNA-synthesizing systems to DNR, altered distribution of DNR within the cell, or altered intracellular metabolism of DNR.

In the present case of DNR resistance developed in vivo, it was possible to distinguish between sensitive and resistant cells by short-term in vitro investigations of explants of the tumor, either of inhibition by DNR of labeling of nucleic acids with radioactive precursors or of drug uptake. It remains to be investigated whether this will also be the case in clinical resistance to DNR.

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