Avarol-induced DNA Strand Breakage in Vitro and in Friend Erythroleukemia Cells

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ABSTRACT

The hydroquinone-containing cytostatic compound avarol inhibits predominantly growth of those cell lines which have a low level of superoxide dismutase. The substrate of this enzyme, the superoxide anion, was found to be formed during the in vitro oxidation reaction of avarol to its semiquinone radical in the presence of oxygen. Under the same incubation conditions plasmid DNA (pBR322) was converted from the fully supercoiled circular form mainly to the nicked circular form, indicating that the compound causes primarily single-strand breaks. Using Friend erythroleukemia cells (FLC) it was found that avarol induces a dose-dependent DNA damage; the maximum number of DNA strand breaks was observed at 5 h after addition of the compound to the cells. Removal of avarol resulted in a rapid DNA rejoining with biphasic repair kinetics (first halftime, 8 min (90% of the breaks) and a second half-time, 40 min (10% of the breaks)). When the degree of avarol-induced DNA damage in FLC was compared with the drug-caused inhibition of cell growth a close correlation was established. Avarol displayed no effect on dimethyl sulfoxide-induced erythroid differentiation of FLC as determined by the benzidine reaction and by dot blot hybridization experiments. From incubation studies of FLC with [3H]avaron as hint was obtained for the formation of an adduct between DNA and the compound. The subcellular distribution of [3H]avaron was studied in liver cells after i.v. application of the compound. The predominant amount of the compound was present in the cytosolic fraction; little avarol was associated with plasma membranes, nuclei, and mitochondria. Using (a) oxidative phosphorylation and (b) oxygen uptake as parameters for mitochondrial function, no effect of the compound on the activity of this organelle was determined.

These results suggest that avarol forms superoxide anions (and in consequence possibly also hydroxyl radicals) especially in those cells which have low levels of superoxide dismutase. Moreover, evidence is provided that the active oxygen species cause DNA damage resulting in the observed cytotoxic effect.

INTRODUCTION

Avarol (Fig. 1, I) is a sesquiterpenoid hydroquinone (1, 2) which has been isolated in large quantities from the sponge Dysidea avara (2). Recently it was found that the compound displays a strong cytostatic activity against the lymphoma cell L5178y (3) and a lower antiproliferating activity towards human and murine lymphocytes (4). Moreover it was established that avarol as well as its quinone derivative avarone shows a selective antiviral activity in the human immunodeficiency virus (human T-cell leukemia virus IIIa) Hg cell system (5). On the subcellular level, avarol/avarone acts as an antimotic agent (6) and causes an inhibition of SOD in vitro as well as in an intact cell system (7).

It is the aim of the present investigation to elucidate that molecular mechanism which is responsible for the cytotoxicity of avarol. Together with earlier data (7), which showed that avarol is converted into its corresponding quinone derivative avarone (Fig. 1, II) via the semiquinone free radical, we provide experimental evidence that superoxide radical anions (O2-) are produced by avarol in the presence of oxygen (O2). This radical species and/or hydroxyl radicals (OH'), which may be formed from O2 and H2O2, induced DNA damage both in vitro and in FLC. The interaction of these oxygen radicals with DNA, resulting in the formation of DNA breaks, has been documented earlier (8). Simultaneously, we studied if the drug-free superoxide radical anions could be detoxified within the cells by the SODs. In view of published data (9), that free radicals are involved in the process of cellular differentiation and derepression of genes, we studied the influence of avarol on the differentiation of FL cells. It is known that a series of metabolic inhibitors are potent inducers of erythroid differentiation in FL cells (10). Finally, we studied the effect of avarol on mitochondrial function. This series of experiments was necessary in view of our previous findings (7) in which a half-wave potential [E1/2] for avarol/avarone, ranging from -210 mV (at pH 10) to +185 mV (at pH 3), was determined. Hence an interaction of avarol/avarone with enzyme complex(es) of the respiratory chain appeared to be possible.

MATERIALS AND METHODS

Materials. Hexokinase (from yeast; 140 units/mg) was obtained from Boehringer, Mannheim (Germany); nitro blue tetrazolium was from Sigma, St. Louis, MO.

Superoxide dismutase (from beef erythrocytes; specific activity, 300 units/mg) was a gift of Firma Grünenthal GmbH, Stolberg (Germany).

Avarol was isolated from the sponge Dysidea avara as described (2). For the in vitro studies avarol was dissolved at a concentration of 10 mg/ml in a solvent system consisting of 60% glycerol (water free), 100% distilled water, and 969 g of polyethylene glycol 400. For the cell culture experiments, the compound was dissolved in Me2SO (10 mg/ml). The maximum concentration of Me2SO used was 0.1%; at this concentration the solvent was without influence on cell growth or the state of differentiation. [3H]Avarol was obtained by catalytic exchange with tritium-labeled water as described (11). The material was purified by high-pressure liquid chromatography (12); the specific radioactivity was 24.2 Ci/mmol.

Analysis of DNA Strand Breakage in Vitro. The potential of avarol to cause DNA strand breakage was assayed by its ability to induce the relaxation of negatively supercoiled plasmid pBR322 (13). The reactions were terminated by adding 10 mM EDTA and 0.1% sodium dodecyl sulfate (final concentrations). The reaction products were analyzed by electrophoresis in 1% agarose gels followed by ethidium bromide staining and visualization with UV (300 nm).

The standard assay mixture contained in a final volume of 20 μl, 0.3 μg of negatively supercoiled circular pBR322 DNA (containing less than 10% nicked circles) in 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl. 15 μg/ml of bovine serum albumin and avarol at different concentrations. Reactions were performed at 30°C for 0–30 min, under either nitrogen or oxygen while shaking.

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The abbreviations used are: SOD, superoxide dismutase; FLC, Friend erythroleukemia cells; Me2SO, dimethyl sulfoxide; EDTA, concentration which causes a 50% inhibition of cell growth in vitro, when compared with the control; P/O (ratio), atoms of P; esterified per atom of oxygen uptake.
As a control, pBR322 DNA was incubated in the DNA topoisomerase II assay (with 1 mM ATP), using 2 μg/20 μl of hen oviduct matrix-associated topoisomerase II as enzyme source, essentially as described (14). The incubation was performed for 30 min at 30°C.

Determination of Superoxide Anions. For the detection of superoxide anions, the procedure described by Beauchamp and Fridovich (15) was used. This method is based on the conversion of nitro blue tetrazolium to blue formazan. The reduction was recorded at 560 nm. The reaction mixture (final volume, 0.5 ml) contained 0.14 M NaCl, 0.01 M sodium phosphate buffer (of different pH), 0.15 mM nitro blue tetrazolium, and different concentrations of avarol. Where indicated, superoxide dismutase was also added to the assay. The reaction was performed at 37°C for 0–10 min (linear part of the kinetics) while shaking under air conditions. In one series of experiments, the reactions were performed under nitrogen.

Culture of FLC. FLC derived from a clone of Friend virus-transformed 745A cells (16) were grown in Joklik minimal essential medium (without NaHCO3, but with 15 mM 4-(2-hydroxyethyl)-l-piperazine-ethanesulfonic acid) supplemented with 10% fetal calf serum. The cells were routinely seeded at 105/ml and incubated at 37°C for 72 h; controls showed a generation time of 11.4 h. Cell growth was determined by cell count with a Cytocomp counter (128-channel counter, Michaelis system).

For the dose-response experiments to determine effects on cell growth, 5-ml cultures were initiated by inoculation of either 104 or 5 × 105 cells/ml and were incubated at 37°C for 72 and 24 h, respectively. The ED50 values as well as the parameters describing the cell distribution curves (mean values) were estimated as described (4). The values presented are means of 10 parallel experiments each.

The effect of avarol on the Me2SO-induced differentiation of FLC was studied under the following routine conditions. The cells were seeded at 105/ml and incubated for 120 h in the presence of 2.0% Me2SO (10).

Culture of Other Cells. The descriptions of the culture procedures for the following cell lines were given earlier (3, 17): L5178y mouse lymphoma cells; HeLa cells (ATCC CCL 2.2); human malignant melanoma cells (ATCC CRL 1424); human fibroblasts (obtained from foreskin), and macrophage-containing lymphocytes (from mouse spleen).

Assay of Hemoglobin-producing FLC. The number of differentiating (hemoglobin-producing) cells was determined by scoring benzidine-positive cells (18). Five–10 min after addition of the benzidine reagent, an average of 300 cells was scored by 2 observers for benzidine positivity.

Isolation of RNA and Dot Blot Hybridization. After the 120-h incubation period of FLC in the presence of different concentrations of avarol, the cells were harvested and the RNA was extracted, essentially as described (19).

The dot blot hybridization assay was performed according to the method of White and Bancroft (20) with the modifications described in (21). Briefly, RNA was denatured by adding 7.5% formaldehyde and 6× standard saline citrate (0.15 M sodium chloride; 0.015 M sodium citrate, pH 7.4) and heating to 56°C for 20 min. Aliquots obtained after adjusting the sample to 13× SSC were applied to nitrocellulose sheets. The baked sheets were hybridized with the 32P-labeled β-globin-specific probe as described by Maniatis et al. (22) using a manifold suction device (Schleicher & Schuell, Dassel, Germany). For this study a plasmid containing the clone of the 390-base pair restriction fragment D (which covered most of the 3′ exon of the murine major β-globin gene) was used (23). Exposition of the dry nitrocellulose filters to Kodak XAR-5 X-ray film (Eastman Kodak) backed by one intensifying screen was done at −70°C for 2–8 days. The concentration of RNA was determined spectrophotometrically (1 A260 = 40 μg/ml).

Assay for DNA Strand Breakage in FLC. DNA damage analysis was carried out using a fluorometric technique that measures the rate of unwinding of cellular DNA on exposure to alkaline conditions (24). In brief, 35 ml of FLC suspension (initial concentration, 5 × 105 cells/ml) were treated with different concentrations of avarol for 0–24 h and then chilled to 0°C; the cells were collected by centrifugation, washed, and distributed equally to a set of 10 tubes. Cells were lysed with a urea/detergent solution. An alkaline solution was added, and DNA strand unwinding was allowed to occur for a 60-min period (22°C). Then, the samples were neutralized and the amount of residual double-stranded DNA was estimated by using ethidium bromide as the fluorescence dye. After calculating the rate of DNA unwinding, the values were converted to the number of strand breaks per cell by reference to the effect produced by γ-rays. The results are given in units of Qd (25), which is a measure of DNA damage; 1 Qd unit corresponds to about 100 strand breaks/cell. Using this approach, single- and double-strand breaks and alkali-labile lesions are detected but are not distinguishable (24).

Search for Possible DNA-Avarol Adducts. FLC were seeded at a density of 105/ml and 10-ml cultures were incubated in the presence of [3H]avarol (10 μCi/ml; 0.41 μM) for 0 and 30 min and 2 and 10 h under standard conditions. Then, the cells were harvested and the DNA was extracted by a hydroxyapatite procedure (26). Samples of the purified DNA were analyzed for DNA content using the fluorescent dye (H33258) adduct method (27). Herring sperm DNA served as a reference standard. Using this procedure, 6.39 μg of DNA were extracted from 109 cells [yield, 94%]. That content of nonextracted DNA in FLC, determined according to the method of Kissane and Robins (28) was set to 100%. The DNA was used either directly or after its enzymatic digestion to deoxyribonucleosides (29) for determination of the possible associated radioactivity.

Subcellular Distribution of [3H]Avarol in Liver Cells of Rats. Rats (Sprague-Dawley; male; 200 g) were given injections of 10 mg/kg of [3H]avarol (94 μCi/mg) into the tail vein i.v. (3). The animals were killed 30 min or 12 h later and immediately used for subcellular fractionation. The procedure of Fleischner and Kervina (30) was used for the isolation of (a) plasma membranes, (b) mitochondria, (c) nuclei, (d) cell supernatant, and (e) microsomes. The isolation of lysosomes and peroxisomes was performed as described ("Addendum" in Ref. 30). The distribution of the organelles and fractions was established by determination of the relative amounts of DNA or of the following marker enzymes, essentially as described (30, 31): 5'-nucleotidase (plasma membrane); DNA (nuclei); succinate dehydrogenase (mitochondria); aryl sulfatase (lysosomes); catalase (peroxisomes); and glucose 6-phosphatase (microsomes). Using this preparation procedure, we obtained 90% of the total protein (present in the homogenate) in the different subfractions.

For determination of the radioactivity, the samples were treated in 30% H2O2 with 5% NH4OH (37°C, 6 h) in combination with aquasol (NEN). Glacial acetic acid (0.01 vol of the scintillant's volume) was added to suppress chemiluminescence of the alkaline solution.

Mitochondria, used for the study of the function of this organelle, were prepared from rat liver (32). The nuclear envelopes were obtained from nuclei by the procedure of Kaufmann et al. (33). SOD Extraction and Assay. The determination of SOD activity was performed basically as described (34) with some modifications given earlier (2). The cells were homogenized and the cell-free extract was collected; then the total SOD activity and the cyanide-insensitive manganese-SOD activity in the presence of 1.0 mm CN− were determined as described (7). The copper/zinc-SOD activity was estimated as the difference between the total and the manganese-SOD activities. The...
specific activity is given in units per mg of protein (34).

Measurements of Mitochondrial Functions. Measurement of oxidative phosphorylation was performed as described (35).

The oxygen uptake was measured by the Warburg manometric method (36). The reaction mixture in a Warburg flask (total volume, 3 ml) had the following composition: 17 mM sodium phosphate buffer (pH 7.4); 5 mM magnesium sulfate; 13 mM DL-β-hydroxybutyrate (substrate); 15 mM glucose; 0.013 mg/ml hexokinase; 2 mM ADP; and 0.5 ml mitochondria suspension (corresponding to 0.5 g of liver). Glucose, hexokinase, and ADP were pipetted into the side arm of the Warburg flask and added after the flask had been connected to the manometer and the reaction mixture had reached 30°C. A 1" zoll filter paper (soaked with 0.2 ml of 2 M NaOH) was placed into the central well of the flask in order to absorb the liberated CO2. Incubation was performed for 15 min at 30°C. Immediately after termination of the incubation, 0.5 ml of the reaction mixture was withdrawn and transferred to the manometer. The oxygen uptake was calculated as described (36). The relation of oxygen uptake (in mole atoms) to ester formation (in mole atoms) was used to calculate the P/O ratio.

Protein was determined according to the method of Lowry et al. (37).

RESULTS

Correlation of Avarol Cytotoxicity with the SOD Content

A comparison of the levels of both copper/zinc- (cytosolic species) and manganese-containing SOD in a series of cell lines with their corresponding sensitivity to avarol shows a striking parallelism (Table 1). It becomes evident that the higher the SOD content the lower is the sensitivity of the respective cell line to the effect of avarol, as shown by the respective ED50 value. From these data, we hypothesize that avarol inhibits cell growth by formation of superoxide anions which are known to cause DNA damage both in vitro (22) and in intact cell systems (38, 39).

Effect of Avarol on DNA in Vitro

Degradation of pBR322 DNA in Vitro. Incubation of pBR322 DNA in the presence of avarol caused a conversion of fully supercoiled DNA (form I) to nicked circular molecules (form II) only in the presence of oxygen (Fig. 2, lanes a, c, and d); only little double-strand broken DNA was detected in the assays containing avarol and oxygen, after an incubation period of 30 min (Fig. 2, lane d; form III). In the presence of nitrogen only minute amounts of nicked circular molecules were formed (Fig. 2, lanes b, g, and h). As a control, pBR322 DNA was incubated with DNA topoisomerase II in order to identify the mode of conversion of plasmid DNA (Fig. 2, lanes e and f).

Table 1. SOD level (both copper/zinc- and manganese-containing species) in different cell lines in comparison with their susceptibility towards an avarol effect

<table>
<thead>
<tr>
<th>Cell line</th>
<th>SOD activity (units/mg protein</th>
<th>Sensitivity towards avarol (ED50 in μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Copper/zinc Manganese (ED50 in μM)</td>
<td></td>
</tr>
<tr>
<td>Friend leukemia cells</td>
<td>64.9 ± 2.5*</td>
<td>43.5 ± 1.3 21.4 ± 1.8 1.61 ± 0.25</td>
</tr>
<tr>
<td>L5178 lymphoma cells</td>
<td>58.3 ± 2.3</td>
<td>39.1 ± 1.2 19.2 ± 1.6 0.93 ± 0.13</td>
</tr>
<tr>
<td>Murine lymphocytes</td>
<td>75.1 ± 2.7</td>
<td>48.4 ± 1.6 26.7 ± 2.3 2.91 ± 0.23</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>104.9 ± 4.2</td>
<td>75.6 ± 2.2 29.3 ± 2.7 12.4 ± 2.2</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>105.2 ± 4.1</td>
<td>70.6 ± 2.2 34.6 ± 2.9 16.9 ± 2.9</td>
</tr>
<tr>
<td>Melanoma cells</td>
<td>117.3 ± 4.6</td>
<td>78.7 ± 2.4 38.6 ± 3.2 37.8 ± 5.9</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Table 2. Formation of superoxide anions by avarol

<table>
<thead>
<tr>
<th>Incubation conditions (standard assay)</th>
<th>pH</th>
<th>Change in absorbance at 560 nm/10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control incubation</td>
<td>8.2</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>Plus avarol, 10 μM</td>
<td>6.0</td>
<td>0.012 ± 0.002</td>
</tr>
<tr>
<td>Plus avarol, 3 μM</td>
<td>7.4</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>Plus avarol, 10 μM and SOD (30 units)</td>
<td>8.2</td>
<td>0.065 ± 0.009</td>
</tr>
<tr>
<td>Plus avarol, 10 μM, under nitrogen</td>
<td>8.2</td>
<td>0.051 ± 0.007</td>
</tr>
</tbody>
</table>

Formation of Superoxide Anions by Avarol. For the determination of superoxide anions generated, a colorimetric method was used (15). As summarized in Table 2, avarol caused a concentration- and pH-dependent increase in absorbance reflecting a reduction of the dye nitro blue tetrazolium by superoxide anions; maximal formation of superoxide was measured at pH 8.2. To elucidate whether the products formed were indeed superoxide anions, the following 2 control assays were performed: (a) the addition of superoxide dismutase to the reaction mixture inhibited the reduction of nitro blue tetrazolium; (b) the performance of the reaction under nitrogen instead of air caused an almost complete inhibition of the reduction rate. From these experiments it must be concluded that avarol forms superoxide anion radicals in aqueous solution.

Effect of Avarol on Erythroleukemia Cells

Influence on Cell Growth and Me2SO-caused Differentiation. When FLC were seeded at a density of 10⁴ cells/ml and then incubated for a period of 72 h, avarol inhibited cell growth to 50% of control (= ED50 value) at a concentration of 1.61 ± 0.25

Fig. 2. Agarose gel electrophoresis of pBR322 DNA following incubation with avarol at a drug to DNA base pair ratio of 1:220 (as described in "Materials and Methods"). Incubation was performed under oxygen (lanes a, c, d) or nitrogen (lanes b, g, and h) for 0 min (lanes a and b) or 30 min (lanes c, d, g, and h). In lanes e and f pBR322 DNA was incubated with DNA topoisomerase (Topo II) (see under "Materials and Methods"). FI, form I, or fully supercoiled circular DNA; FII, form II, or nicked circular molecules; FIII, form III, or linear molecule.
AVAROL-INDUCED DNA DAMAGE

(49) \( (SD) \mu M = 0.51 \pm 0.08 \mu g/ml \). Under the assay conditions used for the determination of the influence of avarol on the integrity of DNA (inoculum, \( 5 \times 10^5 \) cells/ml; incubation period, 24 h), the \( ED_{50} \) was 10.8 \( \pm 1.31 \mu M \).

Avarol, in the absence of \( Me_2SO \), did not cause an induction of FLC erythroid differentiation. The cultures (inoculum, \( 10^6 \) cells/ml; incubation for 120 h) were treated with 0.1, 0.3, 1, 3, and 10 \( \mu M \) of avarol. At the end of the incubation the percentage of benzidine-positive cells was determined. In the avarol-treated cultures less than 5% of the cells were benzidine positive. In the positive control assays, using 2% \( Me_2SO \), 88% of the FLC underwent a differentiation process (Fig. 3). Next, it was studied whether avarol inhibited \( Me_2SO \)-induced differentiation of FLC. The results (Fig. 3) clearly show that the degree of the avarol-caused reduction of the percentage of benzidine-positive cells was directly correlated with the degree of the drug-induced inhibition of cell growth.

The dose-dependent inhibition of FLC erythroid differentiation was further substantiated by analysis of the RNA extracted from FLC treated with avarol in the presence of 2% \( Me_2SO \) (Fig. 4). Using the dot blot hybridization analysis with a \( \beta \)-globin specific probe, a pronounced dose-dependent reduction of the \( \beta \)-globin RNA content was observed. An approximate 50% reduction was observed with RNA extracted from FLC treated with 3 \( \mu M \) of avarol (Fig. 4, lane c).

Production of DNA Strand Breaks in FLC. Avarol, if administered to FLC in culture, rapidly induces strand breaks in intracellular DNA in a concentration-dependent manner (Fig. 5). The DNA damage was determined by a sensitive fluorometric technique (24). The number of strand breaks, expressed in \( Q_d \) units as described (25), reached a maximum 5 h after addition of the compound. After this period of time the numbers were as follows: 5 \( \mu M \), 33; 10 \( \mu M \), 49; and 15 \( \mu M \), 75 \( Q_d \) units.

After a longer incubation period, the number of strand breaks decreased and leveled off after 18–24 h to the following \( Q_d \) unit values (24 h): 5 \( \mu M \), 12; 10 \( \mu M \), 25; and 15 \( \mu M \), 28.

FLC have the ability to rejoin strand breaks rapidly after exposure to avarol (Fig. 6); 50% of the breaks had been repaired already during the first 5 min after removal of the compound, both in the assays with 10 and 15 \( \mu M \); 90% of the breaks were rejoined in less than 30 min. The repair kinetics appears to be biphasic, if the data are plotted semilogarithmically. In the first component (90%) the half-time of the repair kinetics is 8 min, and in the second component (10%) it is 40 min.

Determination of Possible DNA-Avarol Adducts. FLC were incubated in the presence of 0.41 \( \mu M \) [3H]avarol as described under "Materials and Methods." DNA was extracted and used either directly or after enzymatic digestion to deoxyribonucleosides to determine the specific radioactivity. Fifty \( \mu g \) of a DNA preparation from cells, incubated for different lengths of time (30 min to 10 h) in the presence of [3H]avarol, were used for the determination. The results revealed a specific radioactivity of less than 5 dpm/50 \( \mu g \) DNA. From this result we can calculate that less than one molecule of avarol/1.56 \( \times 10^6 \) molecules of deoxyribonucleoside could have formed an adduct.

Even after separation of the deoxyribonucleosides by high-pressure liquid chromatography as previously described (40),

Fig. 3. Effect of avarol on \( Me_2SO \)-treated FLC. The percentage of benzidine-positive (B+) cells and cell number were determined after 120 h of growth in the presence of 2.0% \( Me_2SO \) with the avarol concentration indicated.

Fig. 4. Dot blot hybridization analysis of RNA from FLC, treated with avarol for 120 h in the presence of 2% \( Me_2SO \). A fixed amount of RNA, 1 \( \mu g \) each, from cultures treated with 0 (a), 2 (b), 3 (c) or 6 (d) \( \mu M \) of avarol was spotted. The RNA was blotted and hybridized with \( ^{32}P \)-labeled plasmid containing a \( \beta \)-globin restriction fragment. The membranes were exposed to X-ray films.

Fig. 5. Effect of avarol on DNA damage in FLC. Cells were inoculated at a density of \( 5 \times 10^5 \) cells/ml and incubated for 0–24 h in the presence of different concentrations of avarol, as described under "Materials and Methods." 0 (○), 5 (×), 10 (□), 15 (○) \( \mu M \) avarol. At the indicated times, samples for analysis of DNA strand-break damage were taken and analyzed for DNA strand breaks (in units of \( Q_d \)). The means of 10 independent experiments are given; the SD was less than 15%.

Fig. 6. Rejoining of DNA strand breaks in FLC during posttreatment of FLC after incubation for 5 h in the presence of 10 (○) or 15 (●) \( \mu M \) of avarol. Further details are given in the legend to Fig. 5. The data of 10 parallel experiments are given. Bars, SD.
we could not determine a significant amount of radioactivity to be associated with the DNA fragments (details not given).

Subcellular Distribution of [3H]Avarol in Liver Cells

Applying the method of autoradiography, we established in a previous study that [3H]a varol accumulates in lymphocytes and in NIH-3T3 cells in the cytoplasmic compartment close to the nucleus (41).

In the present study, we quantitated the subcellular distribution of avarol in liver cells after i.v. injection of the radiolabeled compound into rats. The determinations were performed 30 min and 12 h after injection (Table 3). After these 2 periods of time avarol was found to be primarily localized in the cytosolic fraction (=cell supernatant), 64% with respect to the amount in the homogenate after 30 min and 46% after 12 h, respectively. A considerable amount was also associated with the plasma membranes (30 min, 16.3%; 12 h, 5.1%) and the nuclei (3.9 and 8.6%, respectively). Considering the previous observation that avarol is localized at the periphery of the nucleus (41), the nuclei were fractionated into the nuclear envelopes and the residual nuclear structures. Most of the compound was found in the nuclear envelope-containing fraction (30 min, nuclear envelopes, 83% and residual nuclear structures 13%; 12 h, 79 and 14%, respectively) (100% refers to the total amount of [3H]a varol recovered in the nucleus).

Effect of Avarol on Mitochondrial Function

In order to investigate the effect, if any, of avarol on mitochondrial function, mitochondrial oxidative phosphorylation and oxygen uptake were measured in the presence of varying concentrations of avarol. Avarol, at concentrations below 50 µM had no significant effect on oxidative phosphorylation. In the controls (without avarol), the P/O ratio was 2.96 ± 0.62 (n = 5) and in the assays with 50 µM of avarol it was 2.82 ± 0.58. Only at the high concentration tested (250 µM) was the P/O ratio significantly reduced to 1.60 ± 0.31.

The oxygen uptake rate of the mitochondria remained unchanged at avarol concentrations below 250 µM: controls, 0.72 ± 0.09 nmol O2 consumed/0.5 ml of mitochondrial suspension (containing 1 mg of protein)/15 min; and avarol-treated (250 µM) mitochondria, 0.69 ± 0.10 nmol O2/0.5 ml/15 min. These concentrations of avarol used are 30- to 160-fold higher than those causing 50% inhibition of growth of FLC (see Table 1).

DISCUSSION

It is a characteristic feature of avarol to display a pronounced cell-line dependent antiproliferative effect (3); e.g., growth of L5178y mouse lymphoma cells is inhibited at 90-fold lower concentrations than those required for inhibition of human gingival cells and a 40-fold lower concentration for the same effect on melanoma cells. Evidence has been presented that the antitumor activity of quinone/hydroquinone agents, such as Adriamycin, daunorubicin, trenimon, and aziridinylbenzoquinone is at least partially caused by the production of DNA strand breaks mediated by free radicals and active oxygen species (42, 43). Consequently, we determined the level of the enzyme SOD, which is known to detoxify the superoxide radical anion (O2·) to H2O2 (38), in several different cell lines. The results revealed a clearcut correlation between the level of SOD and the sensitivity of different cell lines to avarol. The higher the SOD concentration, the lower the inhibitory activity of avarol on the respective cell line.

Next we studied whether avarol can form the free oxygen radical species, the superoxide radical anion (O2·), by a one-electron reduction of molecular oxygen (O2); superoxide radical anions are known to be the substrate of SOD (38). From a previous study it was known that avarol is converted to avaron via the semiquinone state (7). This finding already indicated a one-electron transfer mechanism. The data, presented here, unequivocally show that the electron which is liberated during the avarol to semiquinone oxidation process is transferred to oxygen; the resulting superoxide radical anion serves as substrate for the SOD. In the absence of oxygen, no superoxide anions are formed (data given) and also no semiquinone radicals are formed. The finding that superoxide anions are formed from avarol and oxygen does not necessarily mean that this free radical is the ultimate cytotoxic component. It is possible that O2· and H2O2 interact with each other to generate highly reactive hydroxyl free radicals (OH·), as described (44).

Since other quinone/hydroquinone-containing cytostatic agents, e.g., Adriamycin and aziridinoquinone, appear to induce damage in DNA both indirectly by free radical-mediated pathways (45) and directly by binding to DNA, either by intercalation [Adriamycin (46)] or by alkylation [aziridinoquinone (47)] it is important to determine which mechanism is prevalent in the case of avarol. Based on the space-filling model of avarol it appears to be unlikely that this compound binds to DNA by intercalation. Experiments performed in our laboratory confirm this suggestion; avarol (a) does not increase the melting temperature of DNA, (b) does not decrease the buoyant density of DNA, (c) does not increase the intrinsic viscosity of DNA, and (d) does not change the sedimentation coefficient of DNA. The data given herein show that [3H]a varol does not interact with DNA after incubation of FLC together with the compound.

In the central part of this study, it is demonstrated that avarol causes DNA strand breaks both in vitro and in FLC. This effect is not caused by a direct influence but is mediated by superoxide anions or hydroxyl radicals as can be deduced by the plasmid nicking assay. The DNA lesions formed are primarily single-strand breaks as can be deduced from the migration behavior of the modified plasmids during agarose gel electrophoresis. Mainly single-strand broken (relaxed) DNA (form II) and very little double-strand broken (linear) DNA (form III) were detected after incubation of pBR322 DNA with avarol. The DNA strand-break damage is also observed if FLC were incubated with avarol in vitro. Even after the short incubation period of 5 h, a drastic increase of the number of intracellular DNA strand breaks occurs. The breaks were rapidly rejoined after removal of the compound from the cultures. From the biphasic repair kinetics (first half-time, 8 min; second half-time, 40 min) it can
be deduced that 2 DNA repair mechanisms are involved in the rejoining of breaks. It remains to be studied whether the double-strand breaks account for the approximately 10% of strand breaks that are observed to be repaired slowly.

Two lines of evidence were presented suggesting that the DNA-damage induced by avarol is the cause of the drug-induced cytotoxicity: (a) it was determined that the DNA damage was directly correlated with the drug-induced inhibition of FLC growth; and (b) it was shown that avarol had no effect on Me$_3$SO-induced erythrodifferentiation of FLC but interfered with cell growth only.

Previous autoradiographic studies in our laboratory revealed that [3H]avarol is taken up by cells and accumulates in the cytoplasm around the nuclear membrane (41). The data presented here, applying the technique of subcellular fractionation, confirmed this finding. The predominant amount of [3H]avarol, taken up by liver cells in vivo, was recovered in the cytoplasmic fraction. The portion in the plasma membrane, mitochondrial or nuclear fractions was lower. Functional studies on the effect of avarol in mitochondria in vitro (determination of oxidative phosphorylation and oxygen uptake) gave no hint that the compound alters the rate of ATP synthesis in isolated mitochondria. In view of the finding, avarol to be taken up by a large number of mitochondria, it is suggested that the Superoxide radicals which might be formed from avarol and oxygen are detoxified by the mitochondrial SOD.

In summary, this study demonstrates that avarol forms oxygen radicals during its oxidation to its semiquinone form. The superoxide free anions are very likely involved in the mechanism of avarol-caused induction of the cytotoxicity. This conclusion is based on the observation of a direct correlation between the induction of DNA strand breaks and cytotoxicity. It remains to be investigated, whether the observed anti-human immunodeficiency virus effect of avarol (5) is also due to the avarol-mediated production of superoxide radical anions. This line of study seems to be promising in view of the published data showing that the antiviral state in a cell is correlated with the level of SOD (48).

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AVAROL-INDUCED DNA DAMAGE


Avarol-induced DNA Strand Breakage in Vitro and in Friend Erythroleukemia Cells

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