Inhibition of Carcinogen-inducible DNA Amplification in a Simian Virus 40-transformed Hamster Cell Line by Ethacridine or Ethanol

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ABSTRACT

DNA amplification as a mechanism to increase gene expression has been established as a cause of cytotoxic drug resistance and appears to play a role in tumor cell progression. In order to investigate factors which control the process of DNA amplification we have been using a simian virus 40 (SV40)-transformed Chinese hamster cell line (CO60) as a model system. This cell line can be induced to amplify integrated viral DNA with a variety of agents. In this report the following is shown. (a) Addition of ethacridine, an intercalative compound, or ethanol to the culture media inhibits amplification induced by the alkylating agent N-methyl-N'-nitro-N-nitosoguanidine or by γ-irradiation in a dose-dependent fashion. In the case of N-methyl-N'-nitro-N-nitosoguanidine induction (50 μM), the highest concentrations of ethacridine (40 μM) or ethanol (2% v/v) tested reduced SV40 amplification from about 20-fold to less than 2-fold. (b) Neither substance induces significant amplification when applied alone over a wide range of concentrations (0.01–20 μM ethacridine; 0.001–2% ethanol). (c) Significant inhibition of amplification is achieved with nearly nontoxic concentrations of both substances (10 μM; 1%), (d) Without direct interference with the inducer. It is concluded that ethacridine or ethanol treatment uncouples the toxic effects of an alkylating agent or ionizing radiation from their ability to induce amplification in CO60 cells.

INTRODUCTION

Amplification of specific DNA sequences as a mechanism to increase expression of normal or altered gene products (1, 2) appears to be involved in several steps within the process of oncogenesis. Amplified DNA sequences, including amplified (and overexpressed) oncogenes, are found in an increasing proportion of human malignancies (3). In patients, DNA amplification of tumor cells has been shown to correlate with clinical stage, risk of relapse, survival, and metastatic disease (4, 5). In chemically induced mouse skin carcinomas as an experimental model system, cytogenetic equivalents of DNA amplification (double minutes) have been detected (6). In human and rodent cell lines DNA amplification is associated with tumorigenicity (6–8) and metastatic growth in athymic mice (9). Finally, amplification of “drug resistance genes” represents an important mechanism of cytostatic drug resistance of tumor cells (1, 2, 10).

Although the process of amplification is not yet fully understood at a molecular level, there is ample evidence for its inducibility by chemical or physical carcinogens (11–14), cytostatic agents (14, 15), hypoxic conditions (16), and certain viruses (17, 18). Thus the metabolic inhibitors, alkylating agents, and ionizing radiation which are widely used as cytotoxic agents in cancer therapy may unintentionally contribute to further malignization or development of tumor cell resistance. Therefore, inhibition of amplification events appears to be a promising novel strategy for tumor therapy (and possibly prevention).

In order to investigate factors which control the process of DNA amplification we have been using a SV40-transformed Chinese hamster cell line (CO60) which can readily be induced to amplify integrated viral DNA sequences (12) as a model system. Recently, we reported that inhibition of poly(ADP-ribose)ylation, a post-translational modification of nuclear proteins which is strongly induced by DNA breaks, is associated with an increased amplification response (19). To see whether this is part of a continuous inverse correlation between poly(ADP-ribose)ylation and DNA amplification we attempted to study the effect of higher than normal levels of poly(ADP-ribose)ation. For this purpose two drugs were selected that were known to influence poly(ADP-ribose) metabolism in this direction (20, 21). Here it is shown that both of them, ethacridine (6,9-diamino-2-ethoxyacridine, rivanol), an intercalative agent, and ethanol greatly inhibit carcinogen-induced SV40 DNA amplification in CO60 cells.

MATERIALS AND METHODS

Chemicals. MNNG3 was purchased from Serva, Heidelberg, Federal Republic of Germany, and was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.1% in all the culture media used for experiments with MNNG induction. Ethacridine lactate was a kind gift of Chinosolfabrik, Seeze, Federal Republic of Germany, and was dissolved in water. Ethanol (analytical grade) was purchased from Baker, Deventer, The Netherlands. Ethanol concentrations are expressed as percentage v/v.

Cells. CO60 cells (kindly provided by Dr. S. Lavi, Tel Aviv, Israel) were grown as monolayers in Dulbecco’s modified minimal essential medium (GIBCO, Karlsruhe, Federal Republic of Germany), supplemented with l-glutamine (2 mm), penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% heat-inactivated FCS (Biochrom, Berlin, Federal Republic of Germany) at 37°C in 5% CO2. Routine screening of cells for Mycoplasma contamination was negative.

γ-Irradiation. CO60 cells were trypsinized, suspended in complete medium at 106 cells/ml, and irradiated on ice with a Gammacell 1000 137Cs γ-ray source (Atomic Energy of Canada, Ltd.) at a dose rate of 16 Gy/min.

Quantitative Slot Blot Hybridization. For amplification assays with MNNG induction the procedures of carcinogen treatment, quantitative slot blot hybridization, and determination of amplification factors were the same as described recently in detail (19), with the following modifications: (a) culture media contained 5% FCS throughout all the experiments; (b) the hybridization probes used (cloned SV40 and rat albumin DNA) were labeled with [α-32P]dCTP (Amersham, Braun- schweig, Federal Republic of Germany) using the random priming method (23); and (c) all the hybridizations were carried out as described for the SV40 probe in Ref. 19.

Dispersed Cell Assay. This filter in situ hybridization method was performed essentially as described by Lavi and Etkin (24). Briefly, adherent cells were trypsinized and combined with floating cells. Cells were counted with a hemocytometer and an aliquot of 107 was sucked onto nitrocellulose round filters (Sartorius, Göttingen, Federal Republic of Germany) using a filtration apparatus (Schleicher & Schüll, Dassel, Federal Republic of Germany). Filters were then placed onto Whatman No. 3MM paper presoaked with 0.5 M NaOH/1.5 M NaCl solution.

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1 This work was supported by the Deutsche Forschungsgemeinschaft.

2 Preliminary results of a part of this work have been published (22).

3 The abbreviations used are: MNNG, N-methyl-N'-nitro-N-nitosoguanidine; FCS, fetal calf serum.

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(conditions of prehybridization and hybridization were the same as for in situ cell lysis, DNA denaturation and RNA hydrolysis) for 10 min and subsequently onto Whatman No. 3MM paper presoaked with 1 M Tris-HCl, pH 7.0/3 M NaCl-0.3 M sodium citrate (for neutralization) for 10 min. Thereafter filters were baked at 80°C for 30 min. Conditions of prehybridization and hybridization were the same as for slot blots.

Cytotoxicity Assay. CO60 cells were trypsinized and irradiated as indicated. Then the cells were plated at 500/1000/5000 cells per 5-cm Petri dish (after 0/3.2/8.0 Gy γ-irradiation, respectively) in medium containing 10% FCS and drugs as indicated. After 7 days of incubation at 37°C (without medium change) cultures were fixed and stained with Mayer’s hemalum solution (Merck, Darmstadt, Federal Republic of Germany), and colonies of more than 50 cells were counted. Absolute plating efficiencies of untreated cells ranged between 78 and 83%.

RESULTS

Ethacridine, an acridine derivative with antiseptic properties (structure shown in Fig. 1), is known to intercalate into DNA (25) and to inhibit enzymatic degradation of poly(ADP-ribose), possibly by intercalation into this polymer (20). It is nonmutagenic in the Ames test (25), although conflicting results were reported with an Escherichia coli mutagenicity test (26). Ethanol, on the other hand, was found to increase the specific activity of poly(ADP-ribose) polymerase in intact cells (21).

In a first set of experiments SV40 DNA amplification was induced with MNNG (50 μM), an alkylating carcinogen. In order to see whether ethacridine or ethanol has an effect on MNNG-induced amplification, increasing concentrations of these drugs were added to the cultures immediately after the MNNG treatment. Fig. 2 shows slot blot autoradiograms and the respective amplification factors calculated from the hybridization intensities of the SV40 and albumin probes. Since albumin sequences are not amplified under these conditions, they serve as an internal control for the amount of cellular DNA bound to the filter (19). MNNG treatment alone leads to about 20-fold amplification of SV40 DNA sequences within 4 days after treatment. Posttreatment with ethacridine or ethanol inhibits MNNG-induced SV40 DNA amplification in a dose-dependent fashion, with a very marked inhibition (less than 2-fold amplification) at the highest concentrations tested.

The inhibitory effect of ethacridine or ethanol is not restricted to induction of amplification with MNNG. Fig. 3 shows dispersed cell assay autoradiograms of cells that were induced to amplify SV40 DNA by γ-irradiation (31 Gy). This assay allows the detection of single amplifying cells which appear on the autoradiogram as individual spots on a homogeneous background of nonamplifying cells. Again, ethacridine or ethanol posttreatment leads to a strong inhibition of γ-ray-induced amplification.

In order to definitely exclude a direct interference of ethacridine or ethanol with the inducing agent, the time interval

![Fig. 1. Chemical structure of ethacridine.](image_url)

between induction with MNNG and addition of ethacridine or ethanol was increased. The amplification factors given in Table 1 show that ethacridine (20 μM) or ethanol (1%) are inhibitory

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amplification factor</th>
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<tbody>
<tr>
<td>MNNG</td>
<td>40.9/46.1</td>
</tr>
<tr>
<td>MNNG + ethacridine 6 h post-MNNG</td>
<td>3.4/3.0</td>
</tr>
<tr>
<td>MNNG + ethacridine 24 h post-MNNG</td>
<td>9.1/9.1</td>
</tr>
<tr>
<td>MNNG + ethanol 6 h post-MNNG</td>
<td>12.9/10.3</td>
</tr>
<tr>
<td>MNNG + ethanol 24 h post-MNNG</td>
<td>20.7/29.3</td>
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even if added to the cultures as late as 24 h after a 1-h MNNG pulse, i.e., long after MNNG has damaged the cellular DNA. (MNNG degrades almost completely within 1 h in culture medium, data not shown).

Some of the known inhibitors of inducible DNA amplification are able to induce amplification if applied alone, e.g., cycloheximide, aphidicolin, or caffeine (14, 15). Therefore, a possible induction of SV40 amplification in CO60 cells by ethacridine or ethanol alone at various concentrations and times of drug exposure was studied using the very sensitive dispersed cell assay. As is shown in Fig. 4, neither ethacridine nor ethanol induces significant SV40 amplification if present for 4 days in a wide range of concentrations, or if applied for 24 h at 20 μM (ethacridine) or 2% (ethanol), followed by a 3-day release period. A culture treated with MNNG (50 μM) served as a positive control.

The results of cytotoxicity studies are shown in Table 2. Ethacridine (10 μM) or ethanol (1%) slightly reduce the plating efficiency of CO60 cells with or without prior γ-irradiation. These concentrations, however, significantly inhibit SV40 amplification induced by 8 Gy γ-irradiation (data not shown). Whether or not the inhibition of DNA amplification is a consequence of this small increase in cytotoxicity (i.e., by a selective killing of amplifying cells) remains to be seen. However, it should be noted that increasing the toxicity is not necessarily associated with inhibition of amplification, since we could show that 3-aminobenzamide, a drug which potentiates alkylation-induced cytotoxicity, actually enhances SV40 amplification in this system (19).

If ethacridine or ethanol caused a nonspecific reduction of DNA synthesis rates this might explain the effect on amplification, since DNA synthesis is necessary for amplification (15). To exclude this possibility cell proliferation was measured under the conditions used for amplification assays. Unirradiated or γ-irradiated (8 Gy) cells were plated into medium containing ethacridine (10 μM) or ethanol (1%) and growth curves were determined as shown in Fig. 5. With or without preirradiation of cells, both drugs reduce cell proliferation only slightly, consistent with their effect on plating efficiency (Table 2). Thus a nonspecific inhibition of DNA synthesis (and consequently cell growth) can be dismissed as an explanation for the inhibition of amplification.

## DISCUSSION

DNA amplification as a manifestation of genomic instability of tumor cells is an important mechanism in the development of drug resistance and further seems to play a role in tumor progression (1-10). A number of agents have already been reported to inhibit amplification under certain conditions, e.g.,

![Fig. 4. Lack of induction of SV40 DNA amplification in CO60 cells by ethacridine or ethanol (EtOH). Cells were plated at 10^4/75-cm^2 flask. After overnight incubation at 37°C drugs were added as indicated. For the release protocol, 24 h later drugs were removed by rinsing the cultures three times and feeding with fresh medium. All the cultures were harvested 4 days after addition of the drugs and processed for the dispersed cell assay. Cells (10^4) were applied to nitrocellulose filters in duplicate, respectively. Autoradiograms of SV40 hybridizations are shown. R, release.](https://example.com/fig4)

![Fig. 5. Proliferation of CO60 cells after ethacridine or ethanol treatment. CO60 cells (with or without prior 8 Gy γ-irradiation) were plated at 3 × 10^4 [nonirradiated cells (A)] or 6 × 10^4 cells [irradiation (B)] per 5-cm Petri dish into medium without additions (O), or medium containing 10 μM ethacridine (C), or 1% ethanol (Δ). Seventy-two and 91 h later, respectively, parallel cultures were trypsinized and cells were counted with a hemocytometer. Results are expressed as mean values ± SD (omitted if smaller than symbol size) of triplicates.](https://example.com/fig5)

| Table 2 Plating efficiency of CO60 cells after γ-irradiation and posttreatment with ethacridine or ethanol |
|----------------------------------|------------------|------------------|
|                                   | 0 Gy             | 3.2 Gy           | 8.0 Gy          |
| Ethacridine 0 μM ethacridine      | 100 ± 3.3        | 52.6 ± 1.9       | 12.2 ± 0.5      |
| Ethacridine 3 μM ethacridine      | 99.6 ± 2.7       | 51.1 ± 1.5       | 12.5 ± 0.3      |
| Ethacridine 10 μM ethacridine     | 82.5 ± 7.3       | 49.2 ± 2.2       | 10.9 ± 0.6      |
| Ethanol 0% ethanol                |                  |                  | 15.7 ± 0.4      |
| Ethanol 0.3% ethanol              | 100 ± 1.3        | 64.3 ± 2.4       | 14.4 ± 0.2      |
| Ethanol 1.0% ethanol              | 98.7 ± 1.9       | 64.1 ± 2.4       | 10.9 ± 0.2      |

*Values indicate percentage of plating efficiency (mean ± SD of quadruplicates) relative to controls. For experimental procedures see "Materials and Methods."
inhibitors of DNA replication (15), of RNA and protein synthesis (14), caffeine (14, 15), protease inhibitors (15), butyrate (15), and paroviruses (27). However, many of these agents are toxic or induce amplification when applied alone at lower concentrations or after pulse treatment (14, 15), which may preclude a possible therapeutic application.

The results presented here show that (a) in a cell culture model system addition of ethacridine or ethanol effectively inhibits amplification induced by an alkylating agent or ionizing radiation, (b) neither substance induces significant amplification when applied alone over a wide range of concentrations, (c) significant inhibition of amplification is achieved with nearly nontoxic concentrations of both substances, (d) without direct interference with the inducer of amplification, and without reducing the toxicity of the inducer. Taken together, it appears that in this system ethacridine or ethanol treatment can uncouple the toxic effects of MNNG or γ-irradiation from their ability to induce amplification, the latter being viewed as an adaptive cellular response to genotoxic stress (1, 2).

This study was carried out in one model system for inducible DNA amplification, the hamster cell line CO60. Experiments are now undertaken to see how these drugs affect DNA amplification in other cell lines. In this respect the amplification of authentic cellular genes, e.g., dihydrofolate reductase, the amplification of which confers resistance to the cytosstatic agent methotrexate (1), is of particular relevance. Furthermore, it would be interesting to see how amplification in human cells is influenced by ethacridine or ethanol.

This study was prompted by our initial finding that a competitive inhibitor of poly(ADP-ribosyl)ation increased the amplification response of CO60 cells to MNNG treatment (19). In an attempt to study effects of an enhanced poly(ADP-ribosyl)ation, ethacridine and ethanol were applied to CO60 cells, assuming the same drug activities on poly(ADP-ribose) metabolism in this cell system as were described in vitro (20) or in a different cell line (21). The resulting inhibition amplification at concentrations that should affect poly(ADP-ribose) metabolism (20, 21) is consistent with our hypothesis that poly(ADP-ribose) may have a negative regulatory role in carcinogen-induced DNA amplification (19). Whether ethacridine and ethanol indeed act by alterations of the poly(ADP-ribose) metabolism and not by different pathways is unclear as yet and currently under investigation. These findings, however, along with the elucidation of the underlying mechanisms might provide an access to the therapeutic long-range objective of interfering with amplification events in human malignancies.

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