ABSTRACT

The antineoplastic agents methotrexate and 5-fluorodeoxyuridine induce DNA lesions, although the drugs are not incorporated into DNA. The lesions arise as a result of reduced repair of damage occurring in DNA independently of the treatment with antineoplastic agents. When cells containing DNA lesions are treated with calmodulin inhibitors (W-7, phenothiazine, promethazine), the amount of lesions is increased, in all probability due to reduced DNA repair. This is paralleled by increased growth inhibition. Hence by inhibiting calmodulin, one can modulate the levels of DNA lesions and change the growth-inhibitory effect induced by methotrexate or 5-fluorodeoxyuridine. This strongly supports the importance of this type of DNA lesion in the toxic effects induced by the drugs.

INTRODUCTION

The mechanisms by which methotrexate and 5-fluoropyrimidines induce cytotoxicity have been under debate for some time. DNA lesions appear in cells treated with either drug, and the levels of the lesions correlate with growth inhibition (1, 2). The higher the level of lesions, the higher the growth inhibition. 5-Fluoropyrimidines, as 5-FU, induce DNA lesions via two mechanisms, one involving the incorporation of the drug into DNA and the other not. The second mechanism resembles the mechanism of action of methotrexate, which is also not incorporated into DNA. 5-FdUrd shows only the second type of mechanism (2).

The incorporation of 5-FU into DNA varies between cell lines and is modulated by culture conditions. For example, promyelocytic HL-60 cells incorporate 70 times more drug into DNA than do L1210 leukemia cells (3). The incorporated drug is efficiently excised by the action of uracil-DNA glycolases and dUTP nucleotide hydrolases, leaving gaps or fragments (4). So far it has not been possible to determine the importance of DNA lesions relative to the other two mechanisms known to induce cytotoxicity: inhibition of thymidylate synthase reducing the production of nucleotides and the incorporation of drug into RNA. Drug resistance has been shown to involve the amplification (30 to 100 times) of the gene for thymidylate synthase (5).

The second mechanism to induce DNA lesions, used by methotrexate and 5-FdUrd, is more difficult to explain. Both drugs have it in common that they efficiently reduce de novo nucleotide biosynthesis, methotrexate by inhibiting dihydrofolate reductase, and 5-FdUrd by inhibiting thymidylate synthetase. This results in a shortage of dTPP and of purine nucleotides. The shortage of nucleotides has been proposed to in reduced repair of lesions induced irrespective of the drug treatment (1, 2). These "normally appearing lesions" are induced due to, e.g., misincorporation of uridine during DNA synthesis; deamination of cytosine, adenosine, or guanosine; and loss of bases resulting in apurinic/apyrimidinic sites. The number of lesions that appear per day has roughly been estimated as 10,000 purines and several hundred pyrimidines (6). This proposal is further supported from recent data on hydroxyurea, another inhibitor of nucleotide synthesis, showing progressive accumulation of DNA lesions (7).

We wish to further analyze the second mechanism. The ultimate effect of methotrexate or 5-FdUrd on cells is a balance between the extent of damage inflicted on DNA and the ability of cells to repair it. In this paper we have therefore examined whether it is possible to modulate the appearance of DNA lesions. We report here findings from cells with nonfunctioning calmodulin (a calcium receptor protein).

Calmodulin is involved in cell proliferation. Drugs that inhibit the function of calmodulin arrest cell proliferation (8–11). It has been shown that calcium, calmodulin, and/or calmodulin-regulated proteins are involved in determining the toxicity of antineoplastic drugs in cells treated, e.g., with bleomycin, dacarbazine, vincristine, or antracyclines (12–14).

We show here that inhibition of calmodulin potentiates the growth cytotoxicity induced by methotrexate or 5-FdUrd, which is paralleled by increased levels of DNA lesions.

MATERIALS AND METHODS

Cells. Culturing Conditions, and Labeling with [3H]Thymidine. Human colon adenocarcinoma cells (WiDr) were grown as monolayers in medium containing 10% fetal calf serum (2). The same batch of fetal calf serum was used throughout the experiments.

For experiments the cells were seeded in small culture dishes (35 × 10 mm) containing 3 ml of medium. For steady-state labeling of the DNA, we added 3 μCi of [3H]thymidine (22 Ci/mmol; Amersham, Inc., United Kingdom) for 24 h (1 μCi/ml of medium); the cells were then incubated for 24 h in fresh medium.

Survival of drug-treated cells was determined by an outgrowth method. Portions of treated and untreated cells were cultured for 5 days with daily changes of the medium. The level of survival in treated cells was measured by determining the differences in the numbers of cell doublings in untreated and treated cells (1).

Drugs. Methotrexate, 5-fluorodeoxyuridine, phenothiazine, and promethazine were obtained from Sigma Chemical Co., St. Louis, MO. W-7 and W-5 were obtained from Miles, Inc., New York. Calmodulin was obtained from Pharmacia, Inc., Upplands, Sweden.

Cell Lysis and Agarose Gel Electrophoresis. Cell lysis was performed in the dark at 0°C by the addition of 2.25 ml of 0.03 M NaOH (pH 12.1). After 30 min the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M Na2HPO4. The solution was then made 0.5% with regard to sodium dodecyl sulfate (2, 17, 18).

Agarose (0.75%) flat bed gels were made as earlier described (2). The labeled DNA was separated in agarose gels using an LKB Multiphor electrophoretic system. The gels were cut in 1-mm-thick slices, and the radioactivity was measured in a toluene-based scintillation fluid containing 3% Soluene 100, using a Packard scintillation counter.
RESULTS

To determine the effect of various drugs on DNA, we examined the appearance of alkali-labile DNA in cells treated according to different drug protocols. The presence of DNA fragments is visualized in gel electrophoretic separations after lysing the cells in dilute alkali (19, 20). The alkaline treatment removes macromolecules from the DNA and disrupts the base pair structure of the DNA. However, the DNA strands cannot separate before enough time has elapsed to allow unwinding. The unwinding is initiated at gaps present in the DNA chains. X-irradiation of such drugs as 5-FU or 1β-D-arabinofuranosylcytosine induces gaps and/or alkali-labile regions in the DNA, resulting in an increased number of points to initiate unwinding of the DNA (2). The length of DNA helix that may be unwound at each point has been estimated as 20 kilobases (17, 18).

During the unwinding, replication intermediates and drug-induced DNA fragments are released. When the solution is then neutralized, the fragments remain as single-stranded DNA, whereas the high-molecular-weight DNA renatures and forms double-stranded DNA. The single-stranded DNA fragments can then be separated from the double-stranded high-molecular-weight DNA by gel electrophoresis (2).

Fig. 1 shows the results obtained on cells with prelabeled DNA which were treated for 60 min with either methotrexate (10 μM) or 5-FdUrd (1 mM) and then cultivated in fresh medium for another 24 h. The electrophoresis shows the appearance of DNA fragments at Slices 20 to 30, which does not occur when untreated cells are analyzed. The effects of methotrexate and 5-FdUrd on DNA are very similar. The data have been described in more detail earlier (2).

Treatment with the Calmodulin Inhibitor W-7. W-7 (a naphthalene sulfonamide derivative) is an inhibitor of calmodulin (21). When proliferating cells are treated with W-7 for 60 min, DNA fragmentation is detected when the cells are examined immediately after the drug treatment. The damage is increased in synchronized cells when released into the S phase. In contrast, in growth-arrested cells one cannot detect DNA fragmentation. Furthermore the effect of W-7 on DNA is not seen when cell proliferation is stopped by prolonged treatment with W-7 (24 to 48 h). The data make it very unlikely that W-7 by itself attacks the DNA. A likely explanation for the data is that cell cycle-dependent enzymes are regulated by calmodulin (16, 22).

We now examined cells treated with methotrexate (10 μM) or 5-FdUrd (1 mM) for 60 min and then incubated in the absence/presence of W-7 for 24 h. In drug-treated cells one can detect DNA fragments as slices 20 to 30 in the gel (Fig. 1) (2). Fragmentation does not occur in untreated cells. Furthermore the results show much higher levels of DNA fragmentation in cells postincubated with W-7 (Fig. 2, A and B). The data parallel these on bleomycin (12, 13), indicating also reduced repair of DNA lesions induced by methotrexate/5-FdUrd during post-treatment with W-7 (see "Discussion").

Dose-response experiments were also performed. Fig. 2C shows that, by increasing the concentration of W-7, one can induce higher levels of DNA fragmentation in methotrexate-treated cells. A similar find was obtained for 5-fluorodeoxyuridine (not shown).

Control Experiments. W-5 is an analogue of W-7 with a much less inhibitory effect on calmodulin (21). When cells are treated according to the same protocol as in Fig. 2, A and B, but substituting W-5 for W-7, much less DNA fragmentation results (Fig. 3A shows the data for methotrexate).

We also examined the effect of adding calmodulin (100 μg/ml) to the medium simultaneously with W-7. This should quench the effect of W-7 irrespective of whether calmodulin is taken up in the cell and/or stays extracellularly all the time. Fig. 3B shows that the level of DNA fragmentation is reduced in cells first treated with methotrexate and then incubated with W-7 and calmodulin, as compared to cells postincubated with W-7 only. Hence the addition of calmodulin prevents W-7 from augmenting the effect of methotrexate or 5-FdUrd. Calmodulin by itself does not affect DNA (not shown).

Next we performed experiments where we added Ca2+ to the medium simultaneously with W-7. This should quench the effect of W-7 irrespective of whether calmodulin is taken up in the cell and/or stays extracellularly all the time. Fig. 3B shows that the level of DNA fragmentation is reduced in cells first treated with methotrexate and then incubated with W-7 and calmodulin, as compared to cells postincubated with W-7 only. Hence the addition of calmodulin prevents W-7 from augmenting the effect of methotrexate or 5-FdUrd. Calmodulin by itself does not affect DNA (not shown).

The control experiments with W-5, Ca2+, and calmodulin strongly indicate that the observed effect of W-7 is mediated via calmodulin.

Treatment with Phenothiazine or Promethazine. To support the data obtained with W-7, we have used two other inhibitors of calmodulin, phenothiazine and promethazine (23). Cells with prelabeled DNA were treated for 60 min with the drug (methotrexate, 5-fluorodeoxyuridine) and then incubated for 24 h in the absence or presence of phenothiazine or promethazine. Fig. 4, A and B, shows that the incubation with phenothiazine/promethazine results in higher levels of DNA fragmentation.

Dose-response experiments were also performed. Fig. 4C shows that, with higher concentrations of phenothiazine, there is more DNA fragmentation after incubation with methotrexate. Similar findings were obtained with promethazine as well as 5-fluorodeoxyuridine (not shown).

Growth Inhibition Analysis. Growth inhibition analyses were performed using an outgrowth method (1). Cells were treated with methotrexate or 5-FdUrd for 60 min and incubated in the presence or absence of W-7 for another 24 h. The cells were then washed free of the drugs, and 24 h later the growth capacity

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Fig. 1. Human colon adenocarcinoma cells (WiDr) were grown as earlier described (2). Cells with prelabeled DNA were treated with methotrexate (10 μM) (B) or 5-FdUrd (1 mM) (C) for 60 min. The cells were then incubated for 24 h in fresh medium. Cells not treated with drugs (x). The cells were lysed in dilute alkali (0.03 M NaOH) at 0°C for 30 min, the solution was neutralized by the addition of 0.9 ml of 0.067 M HCl/0.02 M NaH2PO4, and finally detergent was added (2, 15, 16). During the treatment with dilute alkali, unwinding of the DNA occurs and DNA fragments resulting from the drug-treatment are released. The DNA fragments do not renature during the neutralization step and can therefore be separated from bulk DNA by gel electrophoresis. The DNA was separated in 0.75% agarose gels (2). 25, 10, and 2 denote the size (in kilobases) and location of single-stranded DNA markers.
Fig. 2. In A, cells with prelabeled DNA were treated with methotrexate (10 μM) for 60 min and then incubated for 24 h in medium containing W-7 (10 μM) (●) or in fresh medium (○). Cells not treated with antineoplastic drugs but incubated with W-7 for 24 h (×). The cells were lysed, and the DNA was then separated in 0.75% agarose gels. 25, 10, and 2 denote the size (in kilobases) and location of single-stranded DNA markers. For other details see Fig. 1. In B, cells were treated with 5-FdUrd (1 mM) for 60 min and then incubated for 24 h in medium containing W-7 (●) or in fresh medium (○). C, dose-response curves. Cells were treated with methotrexate for 60 min and then incubated for 24 h with W-7 at 10 μg/ml (●), 50 μg/ml (○), or 100 μg/ml (×).

Fig. 3. Control experiments. In A, cells with prelabeled DNA were treated with methotrexate for 60 min and then incubated for 24 h in medium containing W-5 (10 μM) (○), W-7 (10 μM) (●), or in fresh medium (×). The cells were lysed in dilute alkali, and the DNA was then separated in 0.75% agarose gels. 25, 10, and 2 denote the size (in kilobases) and location of single-stranded DNA markers. For other details see Fig. 1. In B, cells were treated with methotrexate for 60 min and then incubated for 24 h in medium containing calmodulin (100 μg/ml) and W-7 (○) or in medium containing W-7 alone (●). C, incubation with Ca²⁺. Cells were treated for 60 min with methotrexate (●) or 5-fluorodeoxyuridine (○) and then incubated with medium supplemented with Ca²⁺ (5 mM) and W-7.
of the cells was measured (this point is 0 h in Fig. 5).

The results in Fig. 5 show increased growth inhibition in cells first incubated with methotrexate or 5-FdUrd and then incubated with W-7, as compared with cells incubated only with methotrexate or 5-FdUrd. Hence the recovery from damage is reduced in cells treated with W-7.

DISCUSSION

We have examined further the mechanism by which DNA lesions are induced, and which does not involve the incorporation of drug (methotrexate/5-FdUrd) into the DNA. The lesions are visualized as DNA strand breaks and in all probability arise through reduced repair of DNA lesions appearing irrespective of the drug treatment (1, 2). This interpretation has been strongly supported by recent data showing progressive formation of DNA lesions during treatment with hydroxyurea (7). The increase in the level of DNA fragmentation with time of

![Figure 4](image4.png)

Fig. 4. In A, cells with prelabeled DNA were treated with methotrexate (10 μM) for 60 min and then incubated for 24 h in medium containing promethazine (340 μM) (○), phenothiazine (10 μM) (□), or in fresh medium (△). The cells were lysed, and the DNA was then separated in 0.75% agarose gels. 25, 10, and 2 denote the size (in kilobases) and location of single-stranded DNA markers. For other details see Fig. 1. In A, cells were treated with S-fluorodeoxyuridine (1 mM) for 60 min and then incubated for 24 h in medium containing promethazine (340 μM) (○), phenothiazine (10 μM) (□), or in fresh medium (△). C, dose-response curves. Cells were treated with methotrexate for 60 min and then incubated for 24 h with phenothiazine at 10 μM (○), 25 μM (□), or 50 μM (△).

![Figure 5](image5.png)

Fig. 5. Outgrowth experiments. In A, cells were treated with methotrexate (10 μM) for 60 min and then incubated for 24 h in the presence (△) or absence (○) of W-7 (10 μM). Control cells (■). Cells incubated with W-7 (△). Irrespective of which protocol was used the cells were then washed free of drugs, and 24 h later the growth capacity of the cells was measured. This point represents 0 h in the figure. In B, the same protocol as in A, but the cells were incubated with 5-FdUrd (1 mM) instead of methotrexate.
incubation with the drugs (methotrexate, 5-fluoropirimidines, hydroxyurea) very likely represents the rate of net accumulation of the unrepaired DNA lesions.

In this paper our experiments involve the modulation of the levels of DNA lesions induced by methotrexate or 5-FdUrd and the corresponding change in the growth inhibition induced by the drugs. We find an increase in DNA breakage which is paralleled by increased growth inhibition. This strongly argues that the presence of lesions induced in DNA without the concomitant incorporation of drug is important in determining the efficiency of growth inhibition.

We modulate the level of DNA lesions by interfering with the function of calmodulin using various drugs (W-7, phenothiazine, promethazine). Control experiments show a dose-response curve as well as no effect of the addition of Ca²⁺ with the calmodulin inhibitor. The effect is, however, prevented by the addition of calmodulin to the medium. The drugs used here interact also with proteins other than calmodulin. The use of different classes of calmodulin inhibitors in conjunction with the control experiments makes it, however, very likely that calmodulin is involved in the process of the repair of DNA lesions.

Calmodulin, a calcium-binding protein, exists at high levels in all cells and is responsible for many of the intracellular actions of calcium. It is believed that calmodulin is involved in the regulation of cell proliferation and that its function is altered in malignancy with reduced dependence on Ca²⁺. Calmodulin antagonists are known to enhance the cytotoxic effect of certain antineoplastic agents (e.g., doxorubicin, vincristine, bleomycin, dacarbazine) (23). Few antineoplastic agents are used as single agents; most are used with other drugs that utilize a variety of different mechanisms to kill malignant cells.

Bleomycin is so far the best examined drug. The mechanism of enhanced cytotoxicity has so far not been clearly established, but it is not due to increased drug accumulation, reduced drug efflux, or altered metabolism (13). Also when calmodulin inhibitors were added, bleomycin À2+-increased breakage of plasmid DNA was not observed, demonstrating that the production of toxic free radicals was not augmented.

The data from the studies on bleomycin suggest that there exists a calmodulin-mediated system of DNA repair. This interpretation is also in good agreement with the present data on methotrexate and 5-FdUrd. The reduced or inhibited DNA repair results in an increase in DNA lesions which is paralleled by increased cell growth inhibition. In agreement when the cells are treated with W-5, instead of W-7, there is no increase in the level of DNA lesions nor in growth inhibition. W-5 is an analogue of W-7 with very little, if any, inhibitory effect on calmodulin. The data support the notion that calmodulin is a potential target for cancer chemotherapeutic agents (23).

Furthermore when multiresistant cells are examined it is found that these cells contain greater concentrations of calcium than nonresistant cells (24). Calcium antagonists have been demonstrated to overcome drug resistance in experimental tumors. So far the main interest has focused on work with verapamil as the calcium antagonist and its interaction with vincristine and anthracyclines (14, 15, 25, 26). The effect of verapamil is probably to increase the accumulation of drugs in cells by blocking drug efflux.

REFERENCES

Increased Growth Inhibition and DNA Lesions in Human Colon
Adenocarcinoma Cells Treated with Methotrexate or
5-Fluorodeoxyuridine followed by Calmodulin Inhibitors

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