Biochemical Pharmacology, Vol. 37, No. 11, pp. 2155–2160, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ADENOSINE-DIPHOSPHORIBOSYLTRANSFERASE INHIBITORS CAN PROTECT AGAINST OR POTENTIATE THE CYTOTOXICITY OF *S*-PHASE ACTING DRUGS

KEVIN MOSES, ADRIAN L. HARRIS and BARBARA W. DURKACZ*
Cancer Research Unit, University of Newcastle upon Tyne, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, U.K.

(*Received* 7 *October* 1987; *accepted* 10 *December* 1987)

Abstract—We have investigated the effect of inhibitors of ADP-ribosyltransferase on the cytotoxicity of a range of *S*-phase acting drugs. Co-administration of 3 mM 3-aminobenzamide (3AB) potentiated the cytotoxicity of TG 2-fold, but had no effect on the cytotoxicity of HU, FdUrd or araC. Higher concentrations of benzamides (e.g. 10–20 mM 3AB) produced a G1-specific cell cycle blockade. This treatment prevented cells entering *S*-phase DNA synthesis and consequently protected against the cytotoxicity of the same *S*-phase acting drugs. Thus, using different treatment regimens with 3AB, it was possible to either potentiate or protect against the cytotoxicity of TG.

The substituted benzamides are competitive inhibitors of the chromatin bound enzyme, adenosine-diphosphoribosyltransferase (ADPRT)† (EC 2.4.2.30) [1]. This enzyme synthesises poly (ADP-ribose) homopolymers, which covalently modify a number of chromatin proteins (see Ref. 2 for review). 3-Aminobenzamide, the most commonly used inhibitor, increases DNA strand break frequency and enhances the cytotoxicity of cells treated with monofunctional alkylating agents (giving dose enhancement factors of 2–5-fold) [3–5]. Therefore a role for poly (ADP-ribose) synthesis in DNA repair has been postulated. The increase in DNA strand break frequency following inhibition of ADPRT has been attributed to a failure of the ligation step [6] and/or activation of nucleases which attack DNA non-specifically [7]. Whether poly (ADP-ribose) synthesis directly modulates the activity of enzymes involved in DNA repair or causes conformational changes in chromatin which indirectly affect repair processes is still in dispute.

Furthermore, several other metabolic changes attributable to the benzamides have been described, and the effects of these on DNA repair processes have not been evaluated. It has been shown that the benzamides (1) inhibit incorporation of radiolabel from glucose, formate and methionine into DNA, although DNA synthesis itself is not impaired [8–10], (2) inhibit adenosine transport [11], and (3) inhibit cellular proliferation [11]. This last effect, described by Purnell *et al.*, requires about 5-fold higher concentrations of benzamides than are required to inhibit DNA repair and enhance cytotoxicity. They have shown that cells treated with 10 mM 3-acetylamidobenzamide (3AAB) arrest in either G1 or G2 phase of the cell cycle, without affecting the capacity of cells to complete *S*-phase DNA synthesis. Although monofunctional alkylating agents can damage DNA at any point in the cell cycle, progress through the cell cycle (specifically through *S*-phase) is required to manifest the enhancement of cytotoxicity by the benzamides [12]. Thus there are potential antagonistic interactions as well as synergistic actions of the benzamides on DNA damaging agents.

The effects of the benzamides on the cytotoxicity of *S*-phase acting drugs (i.e. drugs which inhibit DNA synthesis and/or are incorporated into the DNA during *S*-phase) have not been systematically investigated. This is of interest because drugs such as TG, HU, FdUrd and araC are widely used clinically. Cells treated with these drugs accumulate DNA strand breaks [13–17], whose repair could be inhibited by benzamide treatment.

One of the limitations in the efficacy of *S*-phase acting drugs is the necessity for cells to be undergoing DNA synthesis during the period of exposure. Bagshawe [18] has demonstrated that it is possible to reversibly inhibit DNA synthesis in normal cells using the *S*-phase acting drug hydroxyurea, while resistant cancer cells continue to proliferate, thus making them susceptible targets for pyrimidine analogues which can be incorporated into the DNA. This "reversed role chemotherapy" arises as a result of a selective cell cycle blockade in normal cells, and the infliction of DNA damage in proliferating cancer cells.

Therefore, we were interested whether the ability of the benzamides at low concentrations to inhibit DNA repair, and at higher concentrations to additionally block cell cycle progress, could influence the cytotoxicity of *S*-phase acting drugs. This was investigated using a broad range of *S*-phase acting drugs, including HU, TG, FdUrd and ara-C.

In this study, we found that it was possible both

* To whom correspondence should be sent.

† Abbreviations used: TG, 6-thioguanine; HU, hydroxyurea; FdUrd, 5-fluoro-2′-deoxyuridine; araC, 1-β-D-arabinofuranosylcytosine; 3MB, 3-methoxybenzamide; 3AB, 3-aminobenzamide; 3AAB, 3-acetyl-amidobenzamide; ADPRT, adenosine-diphosphoribosyltransferase.

to protect against the cytotoxicity of *S*-phase acting drugs, and also, in the case of TG, to enhance the cytotoxicity, depending on the treatment regimen used with the benzamides.

MATERIALS AND METHODS

Cell culture and media. CHO-K1 cells were routinely maintained in Hams F10 medium (Northumbria Biologicals, Cramlington, U.K.) supplemented with 5% foetal calf serum, 5% newborn calf serum, glutamine (3 mM) and penicillin, streptomycin, mycostatin (100 Units/ml; 100 μg/ml and 50 Units/ml respectively). Cells were grown as monolayers in Petri dishes (Falcon, Becton Dickinson, Oxford, U.K.) at 37° under 5% CO_2.

Drugs. HU, TG, FdUrd and ara-C were all obtained from Sigma (St. Louis, MO). 3MB (3-methoxybenzamide) was also obtained from Sigma. 3AB was obtained from Pfaltz and Bauer Inc. (Stamford, CT), 3AAB was synthesised according to the method of Purnell and Whish from 3-aminobenzamide [1].

Routinely, stock solutions of TG and FdUrd were prepared by dissolving in 0.01 N KOH and sterilised by filtration through a 0.22 μm filter and stored at −20° for up to one month. HU was dissolved in water, filtered and used the same day. The benzamides were dissolved in medium without serum, filtered and stored at 4° for up to a week.

Drug treatment and survival curves. Cells were seeded in 10 cm Petri dishes at 10^5 per dish. They were incubated for 24 hr to ensure that all cells were in exponential phase prior to addition of drugs.

Drugs were administered to the cells at the concentrations and for the time intervals specified in the figure legends. Plates were then washed with phosphate buffered saline and cells trypsinised and replated at densities to give approximately 100 colonies per plate. Plating efficiencies of control (untreated) cells were about 70–80%. Following a 10–14 day incubation, colonies were fixed in methanol: acetic acid (3:1), stained with crystal violet (400 μg/ml) and counted. Each point on a survival curve represents the average of at least 3 independent experiments. Standard erros (bars) are shown only in experiments where variation from the mean is greater than ±5%.

CHO-K1 cell synchronisation. Exponentially grown cells were used to seed 6-well cluster dishes (3 cm) at 10^4 per well. Following 24 hr incubation, medium was aspirated and cells washed with serum-free medium, and then incubated in serum-free medium for a further 16 hr. Synchronous growth was initiated by replacing the medium with serum containing medium. There was no cell loss due to this treatment. Control cells divided synchronously 20 hr later. Cell growth was monitored by Coulter counter.

RESULTS

HU, TG, FdUrd and ara-C exert their cytotoxicity during *S*-phase, either by incorporation into DNA (TG, FdUrd and ara-C) [13, 16, 19] and/or by depleting available dNTP pools (HU, FdUrd) [20, 21]. This *S*-phase specificity is demonstrated with HU and FdUrd in Fig. 1, where exponentially growing cells were exposed for increasing lengths of time to highly cytotoxic concentrations of the drugs. These triphasic curves could be explained by differential sensitivities of cells in different phases of the cell cycle. Cells in *S*-phase (representing about 50% of the population) would be killed rapidly during a short exposure time (1–2 hr). The remainder of the population would be resistant to further killing, while cells approached the G1-*S* boundary (plateau of curve), but eventually *S*-phase DNA synthesis would occur, killing the remaining cells (giving the third part of the curve after 8 hr).

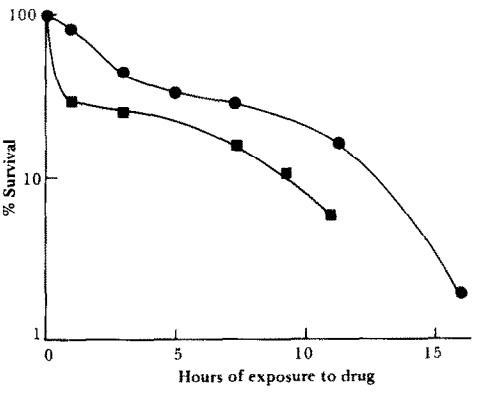

Fig. 1. The effect of the duration of HU and FdUrd treatment on CHO-K1 cytotoxicity. Cells were exposed for increasing lengths of time to either 2 mM HU (●) or 50 μg/ml FdUrd (■) and then plated for survivors in the absence of drugs.

We investigated the effect of treating cells with different concentrations of these drugs for 16 hr in the presence and absence of 3 mM 3AB (which we have shown to inhibit DNA repair and enhance cytotoxicity of monofunctional alkylating agents in this CHO-K1 cell line [unpublished results]). The results are shown in Fig. 2. No potentiation of cytotoxicity by 3AB was obtained with HU, FdUrd or ara-C. However, TG cytotoxicity was potentiated by 3AB with a dose enhancement factor at 10% survival of 2.0.

In order to study the effects of higher doses of ADPRT inhibitors on cell proliferation and antimetabolite cytotoxicity, the effect of increasing doses of benzamides on cell growth were monitored. The resultant growth inhibition curves obtained with 3AB and 3AAB are shown in Fig. 3. It can be seen that 3 mM 3AAB and 20 mM 3AB almost completely inhibited cell growth. The residual cell growth, about 20% of the population, can be attributed to cells which had already passed the benzamide restriction point in G2 and therefore could complete cell division before arresting in the second cell cycle.

We investigated the effect of a benzamide-induced cell cycle blockade on HU cytotoxicity as follows: cells were preincubated with increasing concentrations of benzamides for 8 hr (sufficient time for cells to complete *S* phase DNA synthesis [6 hr]) before addition of 2 mM HU, which reduces survival to 1%, for a further 16 hr. The results are presented in Fig. 4, using 3AB, 3AAB and 3MB. All benzamides produced a dose-dependent increase in the

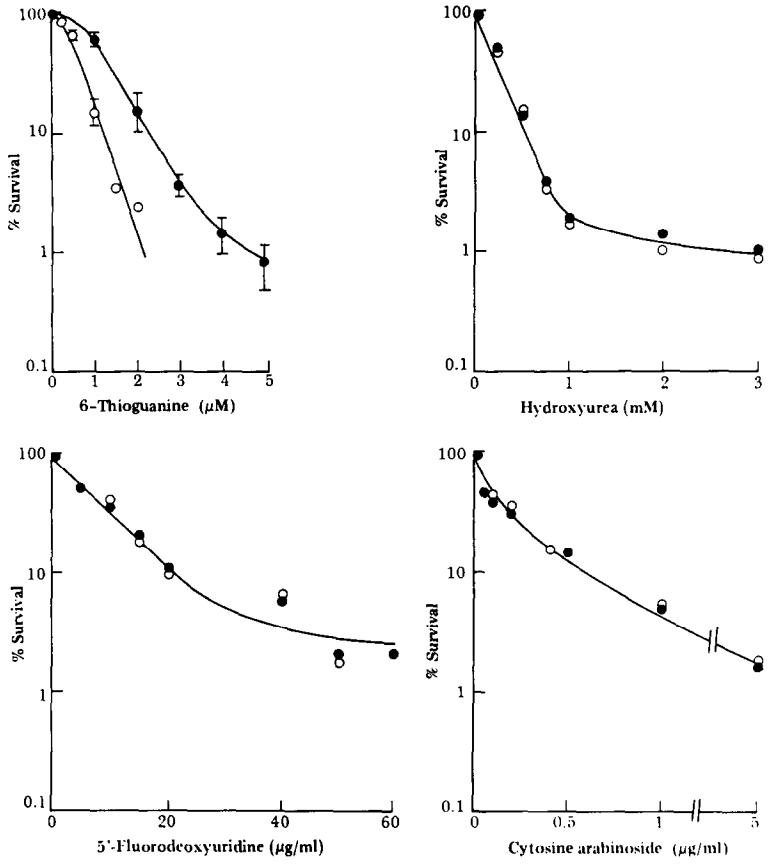

Fig. 2. The effect of 3 mM 3AB on CHO-K1 cytotoxicity of TG, HU, FdUrd and ara-C. Cells were treated with increasing concentrations of the appropriate drug in the presence (○) or absence (●) of 3 mM 3AB for 16 hr. Cells were then plated for survivors in the absence of drugs.

survival of HU-treated cells. For example, the maximum survival obtained with 3AB was 50% at 20 mM, compared to 1% in the absence of 3AB. There is a good correlation between the benzamide concentrations required to produce maximum survival of HU treated cells and those required to inhibit cellular proliferation (compare 3AB and 3AAB in Figs 3 and 4). Furthermore, the potency of the benzamides expressed as the concentrations required to produce maximum protection (3AB, 20 mM; 3MB,

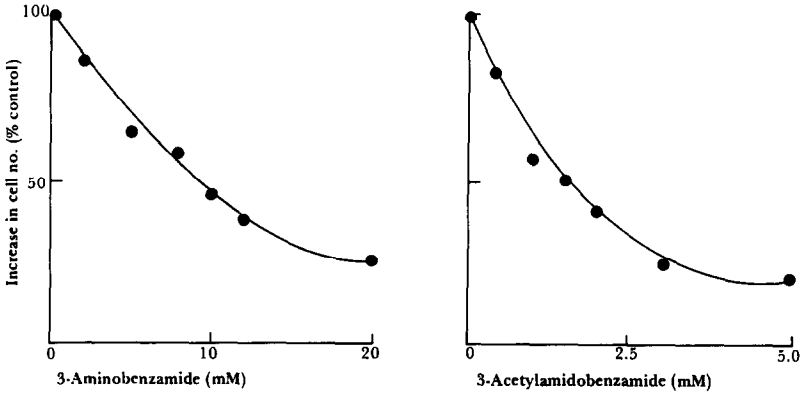

Fig. 3. The effect of ADPRT inhibitors on CHO-K1 cell growth. Cells were seeded at 10^4 per well in 6 well cluster dishes (triplicate samples). 24 hr later, 3AB or 3AAB was added at the concentrations indicated and cultures incubated for a further 48 hr. Cells were trypsinised and cell number estimated by Coulter counting. Relative growth is expressed as % control (drug-free) cell growth.

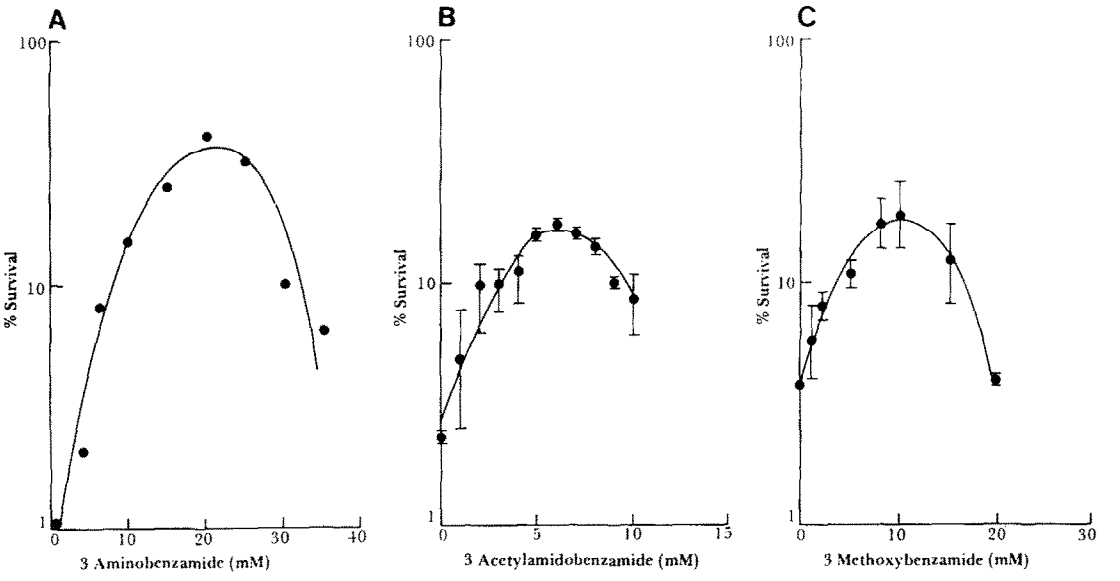

Fig. 4. The effect of preincubation with increasing concentrations of ADPRT inhibitors on the cytotoxicity of 2 mM HU. Cells were exposed to increasing concentrations of ADPRT inhibitors for 8 hr. HU (2 mm) was then added, and incubation continued for a further 16 hr. Cells were then plated for survival in the absence of either drug. ADPRT inhibitors used: A, 3AB; B, 3AAB; C, 3MB.

10 mM; 3AAB, 6 mM) reflects their potency as ADPRT inhibitors (see below). This supports the contention that the protective effect is due to a cell cycle blockade and is mediated via an inhibition of ADPRT activity.

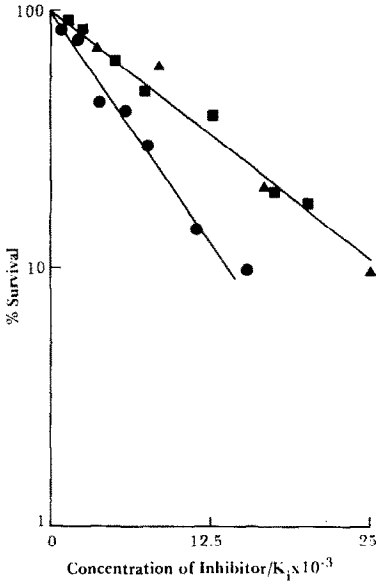

Fig. 5. The effect of a 24 hr incubation with ADPRT inhibitors on cell survival. Cells were exposed to increasing concentrations of ADPRT inhibitors for 24 hr, and then plated for survivors in the absence of drugs. The data has been expressed with respect to the potency of the benzamides as ADPRT inhibitors by dividing the benzamide concentration by its K_i value; ●, 3AB; ■, 3AAB; ▲, 3MB.

The decrease in protection obtained at higher doses of benzamides (e.g. > 20 mM 3AB) can be attributed to the increasingly toxic effects of the benzamides themselves. Figure 5 shows the effect of a 24 hr incubation of CHO-K1 cells in increasing concentrations of benzamides. There was a dose-dependent increase in the cytotoxicity of the benzamides themselves. In general agreement with Purnell *et al.*, there was a good correlation between the potency of the benzamides as ADPRT inhibitors and as cytotoxic agents (K_i values for ADPRT inhibition are: 3AB, 2.6 μm; 3MB, 0.6 μM and 3AAB, 0.4 μM) [11]. However, 3AB was comparatively more cytotoxic, but in our experimental conditions, where the benzamides were exposed to high cell densities, it is possible that the amino group of 3AB is acetylated to produce the more potent ADPRT inhibitor, 3AAB. Presumably, the parabolic curves obtained in Fig. 4 reflect two opposing effects: a cell cycle blockade imposed by the benzamides protecting against HU cytotoxicity, and the benzamides themselves becoming cytotoxic at higher concentrations. This would also explain why survival never reaches 100%, even when cell proliferation is completely blocked by an 8 hr preincubation in high concentrations of benzamides before addition of *S*-phase acting drugs.

In addition, the effects of preincubating cells with increasing concentrations of 3AB on the cytotoxicity of FdUrd and TG were studied. The results in Fig. 6 show that similar protection curves were obtained with TG and FdUrd. Since the range of antimetabolites used exert their *S*-phase cytotoxicity by different mechanisms, it is unlikely that the enhanced survival obtained with 3AB is due to a direct interaction between the drugs.

The results obtained with TG indicate that the timing of administration and the concentrations of

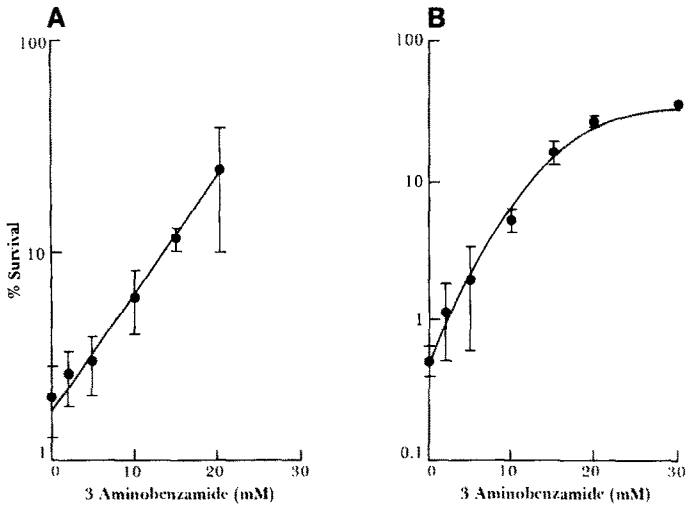

Fig. 6. The effect of preincubation with increasing concentrations of 3AB on the cytotoxicity of TG (5 μM) and FdUrd (50 μg/ml). Experimental protocol used as described in Fig. 4: (A) FdUrd; (B) TG.

benzamides used are crucial to the effect produced on TG cytotoxicity. In the experiments described in Figs 2 and 5, opposing effects of the benzamides of either enhancement of, or protection against cytotoxicity were obtained.

Finally, to assess the position of the G1 block imposed by the benzamides, cells synchronised in G1 by serum starvation were exposed at different times after serum refeeding to 20 mM 3AB, before the addition of 2 mM HU for 16 hr. It can be seen (Fig. 7) that 3AB can protect against HU cytotoxicity if added during the first 2 hr following serum refeeding. If 3AB is added at later times, the protection is rapidly lost, even though there is less contributing cytotoxicity by 3AB itself because of the shorter exposure time to this drug. This experiment positions the 3AB induced G1 arrest at approximately 2 hr after "Start" (defined by the serum requirement to initiate the cell cycle) [22].

DISCUSSION

The results presented here indicate that ADPRT inhibitors can block cell cycle traverse, thereby protecting against the cytotoxicity of *S*-phase acting drugs. Higher concentrations of the benzamides are required to block cell proliferation (e.g. 10–20 mM 3AB) than are required to inhibit DNA repair and enhance cytotoxicity (2–4 mM 3AB). However, even at lower concentrations, a significant proportion of cells are prevented from proliferating, thus altering the cell cycle distribution of the population. We have demonstrated that the benzamides cause a G1 blockade at about 2 hr after "Start". The specific G1 arrest caused by 3AB indicates a possible function for poly (ADP-ribose) synthesis in cell cycle traverse.

Treatment of cells with HU, FdUrd, araC and TG results in a dose-dependent increase in the number of DNA strand breaks, which correlates with increasing cytotoxicity [14–17]. Simultaneous administration with a low concentration of 3AB (3 mM) did not potentiate the cytotoxicity of HU, FdUrd or araC. However, the cytotoxicity of TG was potentiated 2-fold. 3AB has a synergistic effect on cell killing produced by monofunctional alkylating agents and there is evidence that this is due to an effect on the repair of DNA strand breaks. In contrast, the results

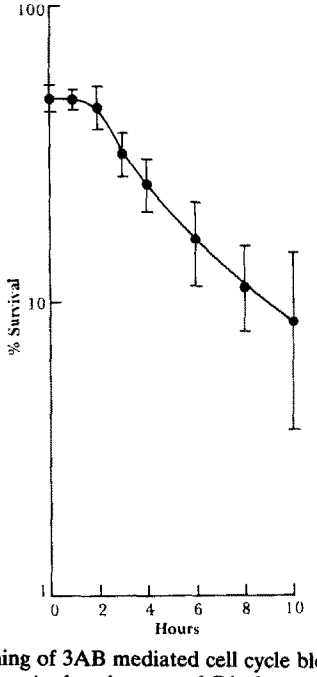

Fig. 7. Timing of 3AB mediated cell cycle blockade. Cells were synchronised at the start of G1 phase as described in Materials and Methods. Cells enter *S*-phase synchronously 10 hr after serum re-addition. 3AB (20 mM) was added simultaneously with serum (0 hr), or at intervals thereafter up to 10 hr. HU (2 mM) was then added simultaneously to all samples and incubation continued for a further 16 hr in the presence of both drugs before plating for survivors. (Horizontal axis: "hours" represents time of 3AB addition following serum refeeding.)

presented here suggest that repair of DNA strand breaks resulting from HU, FdUrd and araC treatment is not modified by 3AB in a way that potentiates cytotoxicity. The mechanism whereby 3AB potentiates the cytotoxicity of TG remains to be elucidated. Inhibition of poly(ADP-ribose) synthesis may affect the repair of TG induced DNA strand breaks. Alternatively, 3AB may affect the uptake or metabolic fate of TG in the cell by a mechanism independent of ADPRT inhibition.

Although co-administration of a low concentration of 3AB potentiates TG cytotoxicity, preincubation of cells with high concentrations of 3AB protects against TG cytotoxicity. For example, 3 mM 3AB produced a 10-fold increase in 2 μM TG cytotoxicity when coadministered, but if cells were preincubated for 8 hr in 20 mM 3AB, the cytotoxicity of 5 μM TG was decreased 40-fold. These diametrically opposing effects, of either potentiation or protection, depended solely on the treatment regimen used with respect to 3AB.

This dual capacity of 3AB to protect against or enhance the cytotoxicity of TG reflects its ability to inhibit both cell cycle traverse and DNA repair. Thus the benzamides could be used for "Reversed Role Chemotherapy" as described by Bagshawe [18], whereby DNA synthesis is prevented in normal tissue, allowing selective incorporation of cytotoxic drugs into the DNA of tumour cells. In this context, it will be of interest to compare the effects of benzamides as inhibitors of both DNA repair and cellular proliferation on transformed and untransformed cell lines.

REFERENCES

1. M. R. Purnell and W. J. D. Whish, *Biochem. J.* **185**, 775 (1980).
2. J. C. Gaal and C. K. Pearson, *Biochem. J.* **230**, 1 (1985).
3. B. W. Durkacz, O. Omidiji, D. A. Gray and S. Shall, *Nature, Lond.* **283**, 593 (1980).
4. M. R. James and A. H. Lehmann, *Biochemistry* **21**, 4007 (1982).
5. J. E. Cleaver, W. J. Bodell, W. F. Morgan and B. Zelle, *J. biol. Chem.* **258**, 9059 (1983).
6. D. Creissen and S. Shall, *Nature, Lond.* **296**, 271 (1982).
7. J. E. Cleaver, K. M. Milam and W. F. Morgan, *Radiat. Res.* **101**, 16 (1985).
8. K. M. Milam, G. H. Thomas and J. E. Cleaver, *Exp. Cell Res.* **165**, 260 (1986).
9. D. J. Hunting, B. J. Gowans and J. F. Henderson, *Molec. Pharmac.* **28**, 200 (1985).
10. K. M. Milam and J. E. Cleaver, *Science* **223**, 589 (1984).
11. M. R. Purnell, W. R. Kidwell, L. Minshall and W. J. D. Whish, in *ADP-Ribosylation of Proteins* (Eds. F. R. Althaus, H. Hilz and S. Shall), p. 98. Springer-Verlag, Berlin (1985).
12. R. J. Boorstein and A. B. Pardee, *J. Cell. Physiol.* **120**, 345 (1984).
13. N. T. Christie, S. Drake, R. E. Meyn and J. A. Nelson, *Cancer Res.* **44**, 3665 (1984).
14. C. R. Fairchild, J. Maybaum and K. A. Kennedy, *Biochem. Pharmac.* **35**, 3533 (1986).
15. J. C. Li and E. Kaminskas, *Cancer Res.* **47**, 2755 (1987).
16. U. Lonn and S. Lonn, *Cancer Res.* **46**, 3866 (1986).
17. R. J. Fram and D. W. Kufe, *Cancer Res.* **42**, 4050 (1982).
18. K. D. Bagshawe, *Lancet* **4**, 778 (1986).
19. D. W. Kufe, P. P. Major, E. M. Egan and G. P. Beardsley, *J. biol. Chem.* **255**, 8997 (1980).
20. L. Skoog and B. Nordenskjold, *Eur. J. Biochem.* **19**, 81 (1971).
21. S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb and J. Lichtenstein, *Proc. natn. Acad. Sci. U.S.A.* **44**, 1004 (1958).
22. L. H. Hartwell, *Bacteriol. Rev.* **38**, 164 (1974).