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Benzamide potentiation of the cytotoxicity of bifunctional galacticol in resistant P388 leukemia correlates with inhibition of DNA ligase II

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Summary. Benzamide (BA) enhances the cytotoxicity of 1,2:5,6-dianhydrogalactitol (DAG) in resistant P388 leukemia cell lines but not in the sensitive parent line. To examine the reason for this difference in response, we carried out an alkaline elution assay using proteinase K to study DNA interstrand cross-linking. At early time points, equal concentrations of DAG produced the same level of interstrand cross-linking (ICL) in the resistant and sensitive P388 leukemic cells, although marked differences were observed in their cytotoxicity toward the two cell lines. In the sensitive cells, neither the amount of DNA cross-linking nor the cytotoxicity changed during the observation period (38 h) in either the presence or the absence of BA. In contrast, the elution rate of the DNA of DAG-treated resistant cells increased with time and had reached the control levels by 38 h. However, when these cells were postincubated with BA for 38 h, the elution rate of DNA was much faster than that observed for the untreated resistant cells, indicating an accumulation of DNA single-strand breaks (SSB). The SSB accumulation caused by BA was associated with an inhibition of the activity of ligase II enzyme, which was stimulated when resistant cells were treated with DAG alone. The potentiating effect of BA on the resistant cells can thus be related to the inhibiting action of BA on the DNA-rejoining enzyme, ligase II. The lack of sensitization by BA of the DAG-treated parent cell line may be attributable to the absence of DNA-SSB formation, which is necessary for ligase II activation through the stimulation of poly(ADP-ribose) synthesis.

Introduction

We have previously reported that benzamide (BA) potentiates the cytotoxic effect of 1,2:5,6-dianhydrogalactitol (DAG) on the DAG-resistant P388 cell line but not on its parental sensitive counterpart [12]. In the presence of BA, significant enhancement in the antitumor activity of alkylating agents, cisplatin, and bleomycin has also been observed by several investigators [4–7, 9, 13, 26, 27]. BA is an inhibitor of poly(ADP-ribose) polymerase (PARP), an enzyme that is involved in the repair of DNA damage induced by some DNA-targeted agents [8, 27]. Although the precise function of PARP in the DNA-repair mechanism is not fully understood, stimulation of PARP has been observed concomitantly with an enhancement of ligase II activity in L1210 cells exposed to dimethyl sulfate, and the enhancement of both of these activities has been prevented by 3-aminobenzamide [8]. These findings prompted us to use PARP inhibitors to sensitize cell lines that had become drug-resistant through an increase in DNA-repair activity.

The alkaline elution technique for single-strand-break measurement and the ligase II enzyme-assay system were used to investigate the nature of the potentiation of DAG toxicity by BA in P388 resistant cells but not in sensitive cells.

Materials and methods

Chemicals and enzymes. DAG was obtained from Chinoin Ltd. (Hungary); BA, alkaline phosphatase (type III-S), polynucleotide kinase, proteinase K, and phenylmethylsulfonyl fluoride (PMSF) were supplied by Sigma; and tetrapropyl ammonium hydroxide was obtained from Eastman-Kodak (USA). Oligo(dT)$_{12-18}$ and poly(dA)$_{255}$ were purchased from Pharmacia (Sweden); hydroxylapatite was supplied by BioRad; and [^{14}C]-methylthymidine, [2-^{3}H]-thymidine, and γ-^{32}P-adenosine triphosphate (γ-[^{32}P]-ATP) were obtained from NEN, Du Pont Ltd. (UK).

Cell survival studies. P388/S and P388/DAG mouse lymphoma cells were established in culture from ascites tumors growing in vivo [2, 21, 22] and were grown in suspension culture containing Fischer's medium supplemented with 10% horse serum. The sensitivity of the cells to DAG and to BA was assessed using the colony-formation assay method. Cells were treated for 1 h (DAG) or continuously (BA) with different doses of the drugs. Untreated and drug-treated cells were plated in plastic petri dishes in medium containing 0.25% agar (Noble agar, DIFCO) and were incubated for 8–12 days in an atmosphere comprising 7% CO_2 in air. Each dose was studied in triplicate dishes, and each experiment was repeated three times. The absolute colony-forming ability of the sensitive

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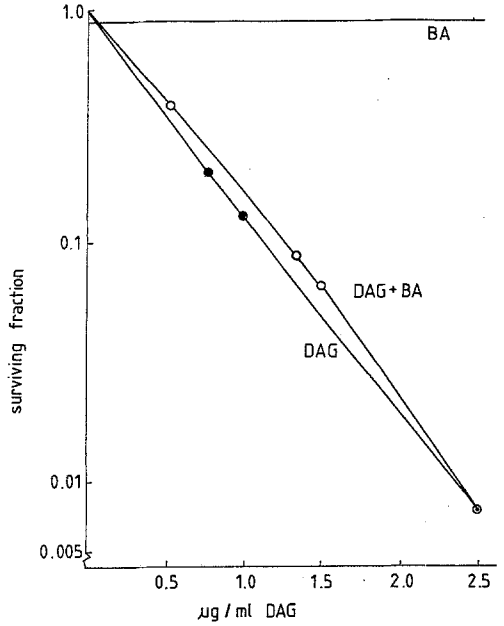

Fig. 1. Effect of BA on the cytotoxicity of DAG toward P388/S cells. Cells were treated for 1 h with DAG and were then grown in the presence and absence of BA. Each dose was studied in triplicate dishes, and each experiment was repeated three times

(P388/S) cells varied between 70% and 90%, and that of the resistant (P388/DAG) cells ranged from about 65% to 70%.

Alkaline elution assay. Exponentially growing cells were template-labeled with [^{14}C]-methylthymidine (0.05 μCi/ml; 52.3 mCi/mmol) or [2-^{3}H]-thymidine (1 μCi/ml; 4 Ci/mmol) for 18 h. Cells labeled with ^{14}C template were exposed to DAG for 1 h, the medium was changed, and the cells were grown in the presence and absence of BA (2 mM) for various periods. Treated cells tagged with ^{14}C (5×10^5) were mixed with approx. the same number of tritiated untreated cells and then exposed to γ-irradiation (5 Gy) at 0° C. The alkaline elution technique developed by Kohn et al. [14], with minor modifications was used, as previously described [1]. Cells were collected on polycarbonate filters (2.5 cm; 2 μm; Nucleopore), washed with phosphate-buffered saline (PBS; 10 ml, pH 7.2, 0° C), and lyzed in 5 ml sarkosinate solution [0.02% sarkosyl, 2 M NaCl, 0.04 M Na^{2+}-ethylenediaminetetraacetic acid (EDTA); pH 10.0] containing proteinase K (0.5 mg/ml, Sigma Chemicals) for 1 h. Then, the filters were washed with Na^{2+}-EDTA.

Alkaline elution was carried out using a mixture of 0.02 M EDTA and tetrapropylammonium hydroxide (pH 12.2). Fractions of eluted DNA (2.0 ml) were collected, and their radioactivity was measured in Acoscint A containing 0.0125 M acetic acid. Radioactivity remaining on the filters was determined as previously described [1]. Elution profiles were obtained by plotting the log-percentage of ^{14}C activity against the log-percentage of tritium levels retained on the filter. The cross-linking factor was calculated using the formula of Kohn et al. [15]:

$$\sqrt{(1-R_o)/(1-R_1)-1},$$

where R_o and R_1 represent the relative retention for untreated and treated cells, respectively. Relative retention was defined as the fraction of ^{14}C-labeled DNA remaining on the filter at the time at which 50% of the tritiated DNA was retained on the filter. Single-strand-break (SSB) frequencies were expressed in rad equivalents as determined from an x-ray elution-calibration curve.

Isolation of ligase enzymes from P388/R cells. First, 2.1 l cell suspension ($2.1-2.6 \times 10^5$/ml) was divided into three portions. Two portions of 700 ml each were exposed to DAG (15 μg/ml) for 1 h, and the third portion served as an untreated control. Cells were then postincubated in fresh medium in the presence and absence of BA (2 mM) for 12 and 24 h, respectively. The isolation of ligases was carried out as follows [17, 23]: after removal of the culture medium, cells were lyzed by sonication (3×15 s at 0° C) in 15 ml buffer containing 0.5 M KCl, 20 mM TRIS-HCl (pH 7.5), 2 mM dithioerythritol (DTE), 1 mM EDTA, and 1 mM PMSF. The lysate was centrifuged at 105,000 *g* for 2 h. All procedures were performed at 2°–4° C.

Protein was precipitated from the supernatant with solid $(NH_4)_2SO_4$ (68%–72% saturation). After centrifugation in a Sorvall centrifuge at 22,000 *g* for 40 min, the protein pellet was dissolved in 5 ml 15 mM potassium phosphate (pH 7.2) and 3 mM 2-mercaptoethanol and then dialyzed against the same buffer overnight. The dialyzed sample was loaded onto a phosphocellulose P11 (Whatman) column (10×1.5 cm) that had been preequilibrated with 15 mM potassium phosphate buffer (pH 7.2). The column was washed with the same buffer, and following the elution of a large amount of protein, the eluent was changed to 15 mM potassium phosphate containing 0.8 M NaCl. Fractions containing ligase enzymes were pooled and put on a column of hydroxylapatite (1×8 cm) that had been preequilibrated with 0.015 M potassium phosphate buffer (pH 7.2). Using stepwise elution with 10 ml each of 0.05, 0.15, 0.4, and 0.5 M potassium phosphate buffer (pH 7.2), 0.5-ml fractions were collected.

^{32}P labeling of oligo(dT). The phosphate termini of oligo(dT) (0.2 μmol/ml) were removed with alkaline phosphatase (type III, 5 units) at 37° C for 30 min [16–18, 20]. After two treatments with the same volume of water-saturated phenol, the water phase was collected and treated twice with the same volume of ethyl ether. Dephosphorylated oligo(dT) (100 pmol nucleotides) was incubated at 37° C with 30 pmol γ-[^{32}P]-ATP (4×10^4 cpm/pmol) in TRIS-HCl buffer (pH 7.5) containing 6 mM $MgCl_2$ and 6 mM mercaptoethanol; 5-unit portions of polynucleotide kinase were added at 15-min intervals until the incorporation plateau had been reached (60–90 min), at which point the reaction was stopped by the addition of phenol. The specific activity found for [5′-^{32}P]-oligo(dT) by this method was $2-4 \times 10^3$ cpm/pmol.

DNA ligase assay. The ligase activity was assayed using a slight modification of Olivera's method [19, 20] as follows. The reaction mixture (200 μl) containing 30 mM TRIS-HCl (pH 7.5), 5 mM $MgCl_2$, 1.0 mM ATP, 5 mM DTE, 200 μg bovine serum albumin/ml, 5 μM poly(dA), 5 μM [5′-^{32}P]-oligo(dT) ($1-3 \times 10^3$ cpm/pmol), and 100 μl enzyme extract was incubated at 37° C for 30 min. The ligase assay is linear with time up to 60 min. Aliquots of 50 μl were removed and boiled for 2 min, and each sample was incubated with 0.1 unit alkaline phosphatase (type III S) at 85° C for 15 min. After the mixture had cooled, 0.2 ml DNA (200 μg/ml) and 0.3 ml 20% trichloroacetic acid (TCA) were added. The precipitate was collected on a 0.45-μm regenerated cellulose filter (Sartorius GmbH, FRG) and washed with TCA (10%) and ethanol. Radioactivity on the filters was measured in Acoscint A. Since alkaline phosphatase is inhibited by inorganic phosphate, fractions showing ligase II activity were pooled and dialyzed against 30 mM TRIS-HCl buffer (pH 7.5), 5 mM $MgCl_2$, and 5 mM DTE, and the ligase activity was assayed as described above. At 37° C, 1 unit of ligase activity catalyses the conversion of 1 nmol [5′-^{32}P]-(dT) into the alkaline-phosphatase-resistant form within 1 h.

Results

Cell survival

Survival curves for both cell lines are shown in Figs. 1 and 2. The doses of DAG producing 50% cell survival were 0.37 μg/ml for P388/S cells and about 10 μg/ml for P388/R cells. No significant difference was observed when P388/S cells were treated with DAG alone or in combination with 2 mM BA. However, the sensitivity of P388/R cells to DAG was significantly increased by 2 mM BA; the ID_{50}

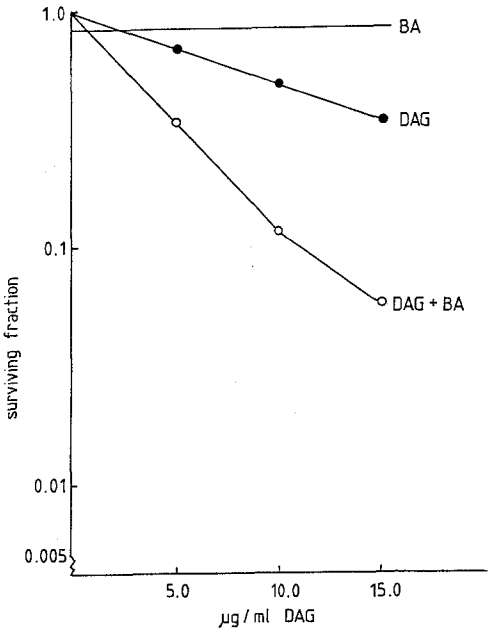

Fig. 2. Effect of BA on the cytotoxicity of DAG toward P388/R cells. Cells were treated for 1 h with DAG and were then grown in the presence and absence of BA. Each dose was studied in triplicate dishes, and each experiment was repeated three times

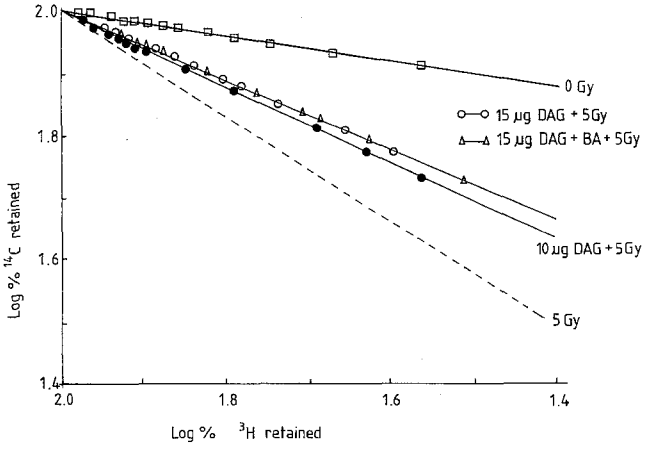

Fig. 3. Alkaline-elution profiles of P388/S cells exposed to DAG (10 and 15 μg/ml) for 1 h and postincubated in the presence and absence of BA for 4 h

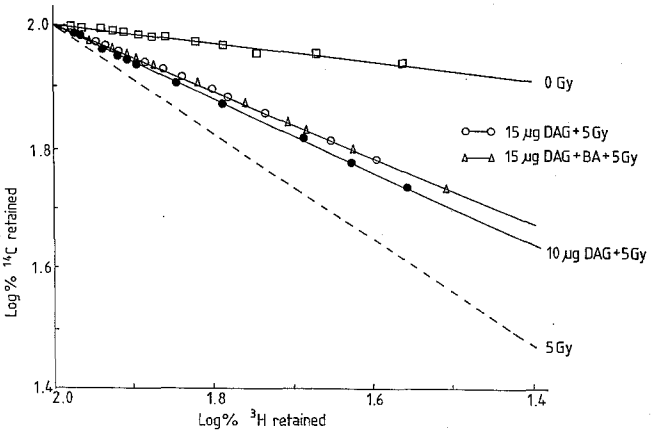

Fig. 4. Alkaline-elution profiles of P388/R cells exposed to DAG (10 and 15 μg/ml) for 1 h and postincubated in the presence and absence of BA for 4 h

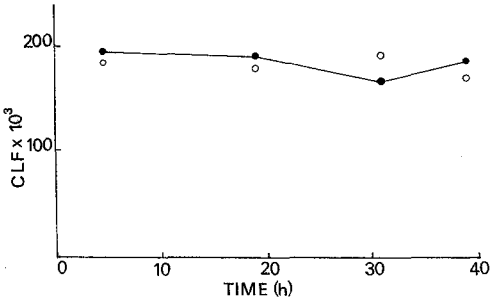

Fig. 5. Time course of the appearance of cross-links in DNA of P388/S cells exposed to DAG (15 μg/ml) for 1 h and postincubated in the presence and absence of BA for various periods

Table 1. Apparent DNA-lesion frequencies in P388/DAG cells treated for 1 h with DAG (15 μg/ml) and postincubated in the presence and absence of BA

Postincubation (h)	DAG		DAG + BA	
	CLF × 10^3	SSB(radEq)	CLF × 10^3	SSB(radEq)
4	197 ± 35	0	195 ± 54	0
18	120 ± 33	0	57 ± 21	0
24	36 ± 12	0	−60 ± 16	56 ± 15
32	−22 ± 4	18 ± 7	−88 ± 21	89 ± 21
38	0 ± 8	6 ± 4	−32 ± 19	124 ± 37

Data represent mean values ± SD for 3 determinations

value (the dose inhibiting the growth of 50% of the cell population) decreased to 5 μg/ml in the presence of BA.

Alkaline elution assays

The alkaline elution studies revealed that at 4 h after their exposure to DAG, both sensitive and resistant cells exhibited the same degree of interstrand cross-link formation (CLF, approx. 196×10^3) at the same DAG dose (15 μg/ml), although this dose elicited markedly different levels of cytotoxicity. The 4 h period of postincubation with BA did not affect DNA interstrand cross-link (ICL) formation in the sensitive or the resistant cells (Figs. 3, 4). The two cell lines, however, showed very different time courses with respect to the removal of ICL. During the observation period, the cross-linking level in the sensitive line did not change considerably in either the presence or the absence of BA (Fig. 5). However, the elution rate of DNA from DAG-treated resistant cells increased with time and was approximately the same as that of the DNA from untreated irradiated cells at 38 h (Table 1, Fig. 6). When the DAG-treated resistant cells were grown in the presence of BA, the elution rate increased more rapidly and had

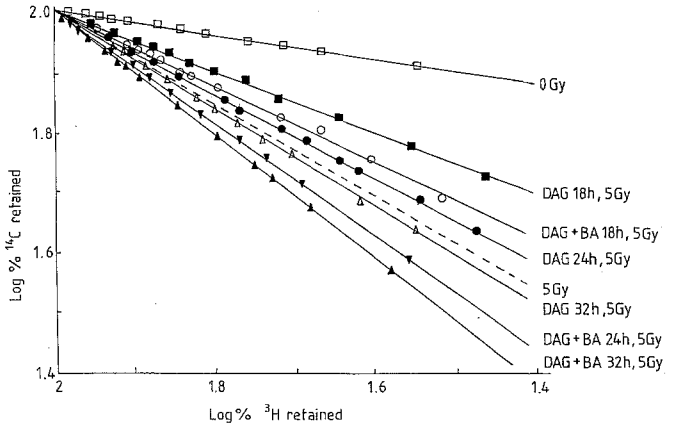

Fig. 6. Alkaline-elution profiles of P388/R cells exposed to DAG (15 μg/ml) for 1 h and postincubated in the presence and absence of BA for various periods

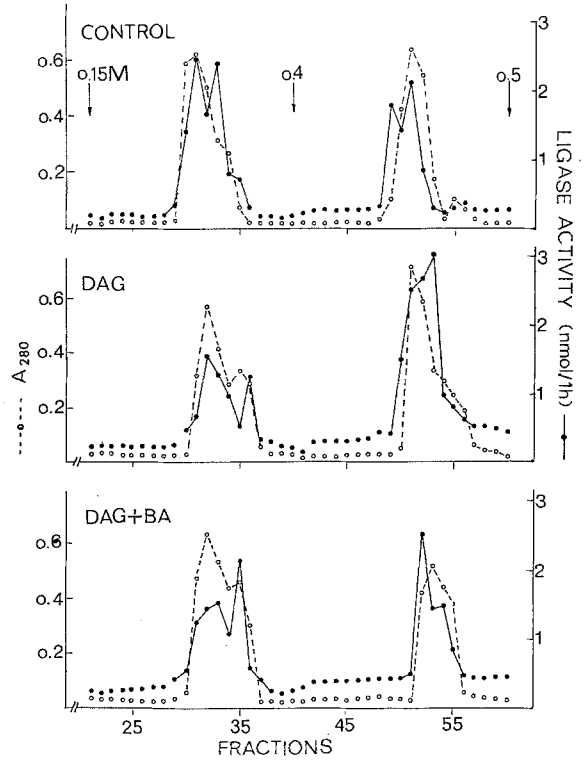

Fig. 7. Stepwise elution of ligase I and ligase II enzymes from the hydroxylapatite column with increasing concentrations of potassium phosphate buffer (pH 7.2). P388/R cells were exposed to DAG (15 μg/ml) for 1 h and postincubated in the presence and absence of BA for 24 h

become much faster than the control value by 32 h, indicating an accumulation of DNA single-strand breaks (SSB, Fig. 6). The time course of the apparent frequencies of DNA lesions caused in P388/R cells by DAG + BA and DAG alone are summarized in Table 1. The actual extent of the lesions may have differed from the values shown, since the cross-links can mask the SSB and vice versa; however, a tendency can clearly be seen, namely, that the repair of DNA lesions in DAG-treated cells has been completed by 38 h but is blocked in the presence of BA.

Table 2. Stimulation of DNA ligase II activity in P388/R cells and its prevention by BA

	Specific activity (nmol h^{-1} mg protein^{-1})	
	12 h	24 h
Control	3.16±0.39	3.48±0.53
DAG	3.79±0.51	7.35±0.81
DAG+BA	3.21±0.70	3.21±0.62

After 1 h exposure to DAG (15 μg/ml), cells were grown in the presence and absence of BA. The enzyme activities represent mean values ± SD for 3 independently isolated and purified enzyme preparations

Measurement of ligase activity

Figure 7 shows the final purification of ligases isolated from untreated P388/R cells and from cells that had been treated with a single 1-h dose of DAG (15 μg/ml) and then postincubated for 24 h in fresh medium in the presence or absence of BA. The protein concentration was followed by the measurement of absorbance units (A_{280}) in the eluent, and the activity of ligases was determined in an aliquot of fractions. Prior to the elution of enzymes, unidentified proteins were removed from the hydroxylapatite column using 0.05 M potassium phosphate buffer as the eluent (data not shown). The peak eluted with 0.15 M potassium phosphate was considered to represent DNA ligase I, the enzyme that functions in semiconservative DNA replication. The second peak eluted with 0.4 M phosphate was believed to represent DNA ligase II, which is involved in DNA-repair synthesis [24]. Since alkaline phosphatase is inhibited by phosphate ions, the fractions containing ligase II were pooled and dialyzed against 30 mM TRIS/3 mM DTE prior to the final determination of enzyme activity. When P388/R cells were grown in the absence of BA after 1 h exposure to DAG (15 μg/ml), the specific activity of ligase II was 2.1-fold that of the untreated cells. However, this stimulation was prevented in the presence of BA (Table 2). Enzyme stimulation by DAG could not be detected after a 12-h period of postincubation in fresh medium alone.

Discussion

As we have previously reported, DAG cross-links DNA, forming 1,6-di(guanyl) galactitol, which persists in the DNA of sensitive cells for some time [10, 11]. This DAG-DNA adduct appears to originate from the interstrand cross-linking (ICL) demonstrated in the present alkaline elution studies. Resistance to DAG seems to be attributable to an increase in DNA repair, since the ICL induced by equal doses of DAG was initially the same in both cell lines and decreased only in the resistant cells with time. The level of ICL induced in the sensitive cells did not change considerably in either the presence or the absence of BA, and the cytotoxicity of DAG toward sensitive cells was also not influenced by BA. In contrast, the cross-linking induced by DAG in the resistant cells decreased gradually, and after the temporary appearance of a small amount of SSB it was completely repaired. In the presence of BA,

however, the removal of ICL was followed by a marked accumulation of SSB, and the sensitivity of the resistant cells to DAG was definitely increased by BA.

Although the exact function of BA in DNA repair is not yet known, some observations suggest that through inhibition of DNA repair, the antitumor effect of DNA-targeted cytotoxic agents can be increased by BA or its several derivatives [8, 27]. BA has been reported to inhibit poly(ADP-ribosyl)ation, which is involved in the in vivo activation of ligase II enzyme in cells exhibiting DNA damage [8]. Ohashi et al. [18] have also assumed that DNA ligase that is dormant under physiological conditions is activated through an increase in poly(ADP-ribosyl)ation in response to DNA breakage. It is noteworthy that highly purified DNA ligase II from calf thymus is not activated but rather inhibited by the poly(ADP-ribosyl)ation reaction in vitro [25, 28]. Creissen and Shall [8] have found that 3-aminobenzamide inhibits excision repair by preventing the stimulation of ligase II activity induced by dimethyl sulfate. Although ICL repair is a more complex process than excision repair, in the present investigation, BA also blocked DNA repair in DAG-treated resistant cells resulting in an accumulation of SSB, and concomitantly inhibited DNA ligase II, which was stimulated when cells were exposed to DAG alone. The stimulatory effect of DAG was approx. 2-fold, whereas Creissen and Shall [8] observed a 7-fold stimulation of ligase II activity in L1210 cells that had been exposed to dimethyl sulfate. Since ligase II activation is strongly dependent on the nicks in DNA, the lower degree of enzyme stimulation observed in our system was presumably attributable to the relatively small number of SSBs introduced into the DNA by a moderately toxic dose of DAG (15 μg/ml), which resulted in a survival fraction amounting to approx. 30% of the cells. These findings suggest that the partial reversal of resistance in P388/R cells may be due to insufficient DNA repair resulting from the inhibition of ligase II activity by BA.

The failure of BA to sensitize the P388/S cells can be attributed to the stability of the ICL due to a lack of DNA-SSB formation, which is necessary for the activation of DNA ligase II via the stimulation of poly(ADP-ribose) synthesis [3].

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