

Loss of Amplified Genes by Poly(ADP-ribose) Polymerase Inhibitors

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A poly(ADP-ribose) polymerase inhibitor, benzamide (BA), was found to induce flat revertants of NIH 3T3 cells that had been transformed by human *Ha-ras*, rat *Ki-ras*, rat *c-ras*, and human *ret-II*. These genes had been amplified in original transformants, but they were completely eliminated by BA. Contrary to this, endogenous activated *Ha-ras* in a human bladder carcinoma cell line, T24, was not eliminated by BA. The gene loss seemed to be restricted to exogenous and/or amplified sequences. BA also eliminated the amplified *c-myc* gene in HL-60 cells, concomitant with differentiation into granulocytes. We demonstrated that the amplified *c-myc* gene was not present as episomes. It is probably present as double minutes or a homogeneously staining region. Dimethylsulfoxide also induced differentiation at a concentration that did not inhibit poly(ADP-ribose) polymerase. The cell lost the *c-myc* gene in association with this differentiation. The amplified *c-myc* gene in a colon adenocarcinoma cell line, COLO 320HSR, and the amplified *mdr-1* gene in an adriamycin-resistant myelogenous leukemia cell line, K562/ADM, were not eliminated by BA. Various poly(ADP-ribose) polymerase inhibitors also eliminated human *Ha-ras* in the NIH 3T3 transformant and the *c-myc* gene in HL-60 cells.

Introduction

Carcinogenesis is a multistep process involving mutation, amplification, loss, and rearrangement of genes. Oncogene amplification is often associated with malignant progression. For instance, amplification of the *c-myc* gene is often observed in advanced cases of uterine cervical cancer (1) and advanced cases of breast cancer (2), while amplification of *N-myc* is often observed in advanced cases of neuroblastoma (3). Recently, we found that poly(ADP-ribose) polymerase inhibitors eliminated the exogenous amplified sequence in NIH 3T3 transformants (4).

Poly(ADP-ribose) polymerase [ADP-ribosyltransferase(polymerizing); EC 2.4.2.30] modifies a number of proteins by transferring the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to the protein and polymerizing it (5,6). Inhibitors of poly(ADP-ribose) polymerase such as benzamide (BA) and 3-aminobenzamide are potent inducers of sister chromatid exchanges (7). These inhibitors also inhibit DNA replication, causing concomitant accumulation of nascent 10 kb DNA (8), inhibition of DNA repair (9), and modulation of cell differentiation (10-12). 3-Aminobenzamide has been shown to enhance alkylating agent-in-

duced amplification of SV-40 DNA integrated into cellular DNA of the host (13). Recently, a mutant of Chinese hamster V79 cells with very low poly(ADP-ribose) polymerase activity was isolated and shown to be a slow grower, to be sensitive to alkylating agents, and to have a much higher background level of sister chromatid exchanges than the parental V79 cells (14). These findings show that poly(ADP-ribose) polymerase is involved in DNA repair and suppression of sister chromatid exchanges.

In this study, we examined the effects of poly(ADP-ribose) polymerase inhibitors on amplified genes using several NIH 3T3 transformants harboring different oncogenes: a human promyelocytic leukemic cell line, HL-60, in which endogenous *c-myc* is amplified, a human colon carcinoma cell line, COLO 320HSR, in which *c-myc* is amplified as a homogeneously staining region (HSR), and a human myelogenous leukemic cell line, K562/ADM, in which the *mdr-1* gene is amplified.

Results

Elimination of Exogenous Transforming Genes

a1-1 Cells are a secondary transformant of NIH 3T3 cells retaining *c-Ha-ras*^{T24} of the T24 human bladder carcinoma cell line. When these cells were cultured in the presence of 5 mM BA, with cell transfer every 4 to 5 days, most cells became flat after 2 to 4 weeks. On

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Southern blot analysis using the v-Ha-ras DNA fragment (BS-9) as a probe, the intensity of hybridization of the human c-Ha-ras sequence in a1-1 cells was four times that of the endogenous mouse c-Ha-ras sequence. Its hybridization intensity did not change during culture for 4 weeks in the absence of benzamide. Addition of 5 mM benzamide to the medium did not change the hybridization intensity of endogenous mouse c-Ha-ras, but specifically reduced that of the exogenous ras sequence (Fig. 1). These results indicated that flat reversion of a1-1 cells was due to loss of the exogenous transforming gene. There are two possible explanations for the effect of BA; one is that BA might cause selection of flat cells that were induced spontaneously, and the other is that BA might cause specific elimination of the exogenous transforming gene. To clarify the action of BA, we examined its effect on a1-1 cells without cell transfer.

a1-1 Cells were plated at a density of 30 cells/10 cm diameter Petri dish, BA was added on day 2, and the cultures were maintained for 2 weeks. The cell morphology of the colonies was then examined. Each colony was found to contain many clusters of flat revertants surrounded by transformed cells (Fig. 2), indicating the induction, but not the selection, of flat cells.

By cloning these cells, five clones each of transformed cells and flat cells were obtained. Southern blotting showed that all five clones of flat cells had lost the activated human c-Ha-ras sequence, whereas all five clones of transformed cells still retained whole copies of activated human c-Ha-ras. No change of the endogenous mouse c-Ha-ras gene was observed in these 10 clones. We also found that BA had no effect on the human c-Ha-ras^{T24} sequence in T24 cells, in which it exists as a nonamplified homozygous sequence. There-

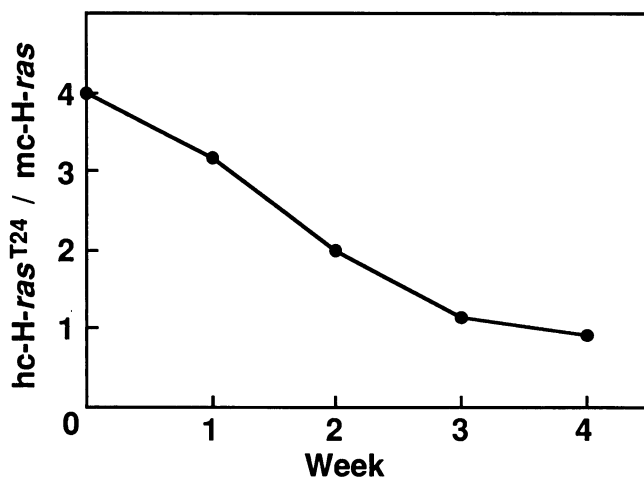


FIGURE 1. Decrease of human c-Ha-ras^{T24} sequence in a1-1 cells by treatment with benzamide. A clone of a1-1 cells was cultured in the presence of 5 mM benzamide for the indicated periods with cell transfer every 4 to 5 days. The hybridization intensity observed on Southern blotting of BamHI digests of cellular DNA with the BS9 fragment of v-Ha-ras as probe is expressed as a ratio to that of the endogenous mouse c-Ha-ras sequence, which was constant.

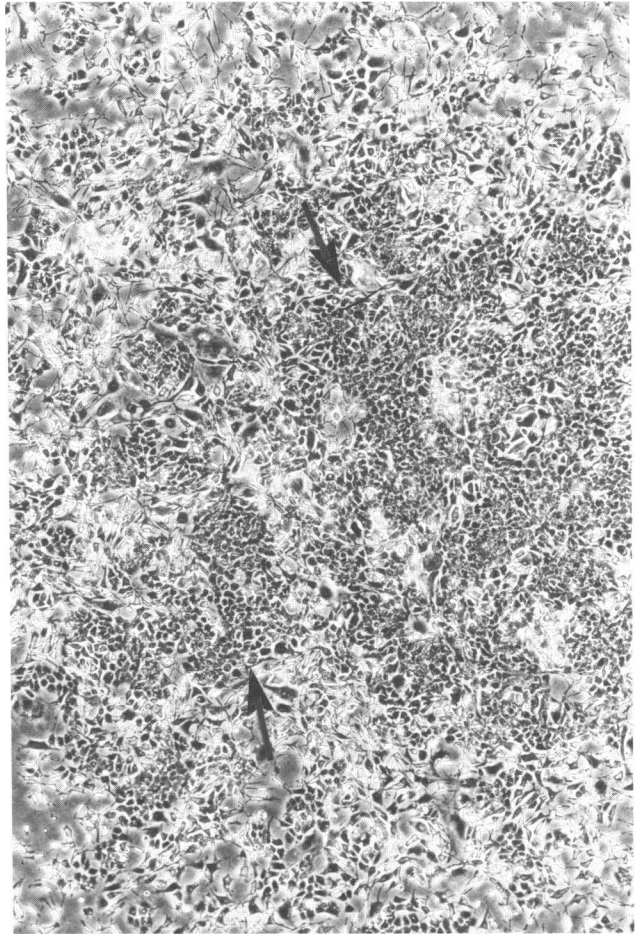


FIGURE 2. Induction of flat cells on treatment with benzamide. Cell morphology of a1-1 cell colony grown in the presence of 5 mM benzamide is shown. Arrows show clusters of flat revertant cells.

fore, we conclude that BA caused elimination of exogenous and/or amplified sequences.

Poly(ADP-ribose) polymerase is composed of three domains, NAD binding, automodification, and DNA-binding domains (6) and has two contiguous zinc-finger structures (15). Not only BA, a competitor with substrate NAD, but also the DNA site inhibitor, coumarin (4), and a newly found potent inhibitor, luminol (4), induced flat revertants efficiently (Table 1). On the contrary, noninhibitory analogues of BA, benzoic acid and 3-aminobenzoic acid did not induce flat revertants of a1-1 cells. Thus, elimination of exogenous amplified sequences seems to be an intrinsic property of poly(ADP-ribose) polymerase inhibitors.

Other transfected genes such as activated Ki-ras, N-ras, c-raf, and ret-II were also very efficiently eliminated by culturing the transformants in the presence of 5 mM BA for 2 weeks (4), and these BA-treated cells were also flattened. These activated oncogenes were of rat (Ki-ras) or human (N-ras, c-raf and ret-II) origin; that is, they were derived from a different species from the host cells.

Table 1. Flat cell induction by poly(ADP-ribose) polymerase inhibitors.^a

Compound	Application, mM	Morphology of colony, %			Plating efficiency, %
		A	B	C	
Benzamide	5.0	8.2	27.4	64.4	27.0
3-Aminobenzamide	5.0	65.8	17.1	17.1	54.1
Coumarin	2.5	13.2	47.3	39.6	33.7
Luminol	1.0	4.4	16.8	78.8	41.9
Benzoic acid	1.0	89.0	3.6	7.3	20.4
	2.5	100	0	0	1.9
3-Aminobenzoic acid	5.0	68.4	28.4	3.2	35.2
Untreated		94.3	3.9	1.8	83.9

^aA clone of al-1 cells was plated at a density of about 90 cells per 10-cm Petri dish and treated with the above compounds for 12 days. Colonies were fixed in methanol and stained with Giemsa's solution. Then they were classified as type A, B, or C by microscopic examination. Type A, a colony composed of only transformed cells; B, a colony composed of less than 50% of flat cells; C, a colony composed of more than 50% of flat cells. Average values for three plates are shown.

Elimination of Amplified Endogenous Gene

To determine the effects of poly(ADP-ribose) polymerase inhibitors on an amplified endogenous sequence, we examined their effects on the *c-myc* gene in HL-60 cells, a human promyelocytic cell line. The human *c-myc* gene is amplified about 16-fold in our stock of HL-60 cells. When HL-60 cells were cultured in the presence of 7.5 mM BA, they differentiated into granulocytes, as reported previously (16). Cell differentiation was determined by examination of nitroblue tetrazolium reducing activity in the presence of phorbol 12-myristate 13-acetate (PMA). As shown in Figure 3, the cells did not grow after differentiation. Scarcely any of the cells reduced nitroblue tetrazolium on day 2, but on day 3 after addition of BA, more than 60% of the cells showed nitroblue tetrazolium reducing activity and appeared granulocytic with segmented nuclei. Treatments with coumarin and 4-hydroxyquinazoline, a newly found potent inhibitor (17), at concentrations of 2.5 mM also induced granulocytic differentiation of the cells after 3 days.

Southern blot analysis showed that the *c-myc* gene

was lost concomitantly with differentiation of the cells on treatment with BA (Fig. 3) and various other poly(ADP-ribose) polymerase inhibitors (17). A noninhibitory analogue of BA, benzoic acid, did not induce either cell differentiation or gene loss (data not shown). We also examined the effect of dimethyl sulfoxide (DMSO), a well-known granulocytic inducer, on *c-myc* gene loss. With 1.6% DMSO, morphological changes and gene loss were observed on day 3 (Fig. 3). DMSO has been found to inhibit poly(ADP-ribose) polymerase *in vitro* at concentrations of the substrate, NAD, of less than 25 nM, but to cause only slight inhibition at cellular concentrations of NAD (K. Ueda, personal communication), which are in the order of several hundred micromolar (18,19). Thus, the mechanism of induction of cell differentiation and gene loss by DMSO is not yet known.

Recently, the amplified *c-myc* gene in HL-60 cells was reported to be present as an episomal element about 250 kb in size at early passages of the HL-60 cells (20). This episomal element can enter agarose on pulsed-field gradient gel electrophoresis (PFGE) (21). Therefore, for determination of the state of the *c-myc* gene in our stock of HL-60 cells, the cells were embedded in agarose

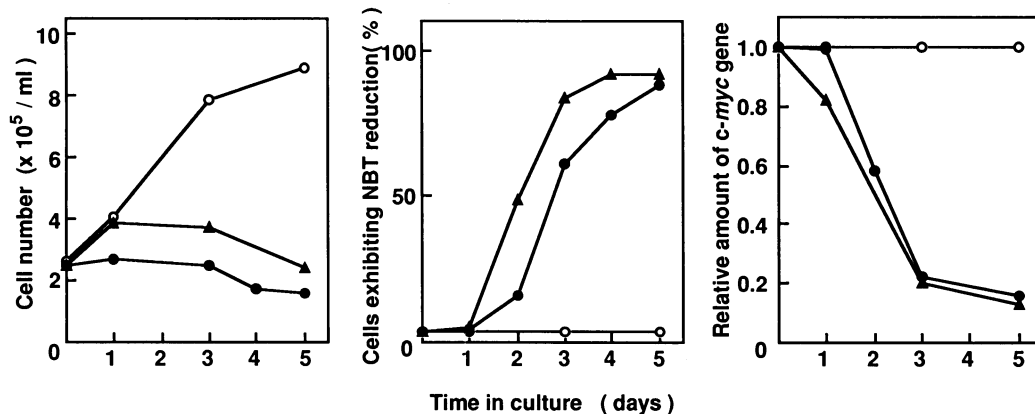


FIGURE 3. Effects of BA and DMSO on HL-60 cells. (Left panel) Cell growth. (Middle panel) Differentiation into granulocytes determined from the nitroblue tetrazolium (NBT) reducing activity. (Right panel) Hybridization intensity of the *c-myc* gene by Southern blot analysis. (●—●) With 7.5 mM BA; (▲—▲) with 1.6% DMSO; (○—○) with no addition.

and lysed with sodium *N*-lauroylsarcosinate, and the uncut DNA was separated by PFGE as described (21). Results showed that the amplified *c-myc* gene in our stock of HL-60 cells was not present as episomes, but possibly present as double minutes (DMs) or a homogeneously staining region (HSR). The HL-60 cells differentiate into macrophages on treatment with PMA or teleocidin, but this differentiation was not associated with gene loss.

Effect of BA on the Amplified *c-myc* Gene in COLO 320 Cells

We also examined the effect of poly(ADP-ribose) polymerase inhibitors on the amplified *c-myc* gene in COLO 320HSR cells. These cells were derived from a human colon adenocarcinoma (22), and induction of their terminal differentiation *in vitro* by poly(ADP-ribose) polymerase inhibitors has so far not been reported. COLO 320 HSR cells were obtained from the American Type Culture Collection (Rockville, Maryland), and a clone was isolated by limiting dilution. A 75-cm² flask was coated with collagen type 1 by adding 7.5 mL of a solution of collagen type 1 (300 µg/mL), standing the flask for 1 hr at room temperature, and then washing it twice with phosphate-buffered saline. About 800 cells of the clone were then inoculated into the flask and cultured in 9 mL of RPMI-1640 medium containing 10% fetal calf serum and 5 mM BA for 12 days.

Under these conditions, cell growth was reduced to about 50% of that in control cultures. In the presence of 5 mM BA, more than 50% of the cells became tightly adherent to the substrate, whereas only a few cells were loosely adherent in the absence of BA (Fig. 4). These adherent cells in cultures with benzamide were flat, clearly differing in morphology from cells in control cultures. Nonadherent cells in cultures with 5 mM BA were removed by pipette with vigorous shaking to separate them from adherent cells, and the DNAs of these nonadherent and adherent cells and also of untreated cells were extracted. Southern blot analysis indicated that the hybridization intensity of *c-myc* was not changed in these flat cells. Thus, the change of cell morphology induced by BA was not associated with loss of the amplified *c-myc* gene.

When the cells were cultured in uncoated flasks, they grew in suspension. Additions of high concentrations of polymerase inhibitors, 5 mM 4-hydroxyquinazoline or 12.5 mM BA, inhibited cell growth almost completely, but no loss of the *c-myc* gene was found after 20 days of treatment with either of these inhibitors. These results indicate that the amplified endogenous *c-myc* gene is not necessarily eliminated by poly(ADP-ribose) polymerase inhibitors.

Effect of BA on the Amplified *mdr-1* Gene in K562/ADM Cells

Serious problems in cancer therapy are resistance of progressed cancer cells to chemotherapeutic agents and

the acquisition of resistance during treatment. We examined the effect of poly(ADP-ribose) polymerase inhibitors on the amplified *mdr-1* gene induced by adriamycin treatment in myelogenous leukemia K562 cells, K562/ADM (provided by T. Tsuruo, University of Tokyo) (22). We isolated three clones of K562/ADM, K562/ADM-1, K562/ADM-2, and K562/ADM-3, and two clones of K562, K562-1 and K562-2. Southern blot analysis of digest of these DNAs with *Hind*III, using pMDR1 (provided by I. B. Roninson, University of Illinois) as a probe indicated that K562/ADM-1~3 have an amplified *mdr-1* gene, as shown in Figure 5. When these cells were treated with various concentrations of BA, their growth rates were reduced dose dependently, about 50% growth inhibition being observed with 5 mM BA. This dose-dependent inhibition of cell growth by BA suggests that BA was taken up into these cells in spite of the presence of the amplified *mdr-1* gene. Figure 5 shows that there was no loss of the *mdr-1* gene even after culture for 5 weeks in the presence of 5 mM BA.

Discussion

Poly(ADP-ribose) polymerase inhibitors were found to eliminate exogenous and amplified sequences efficiently. In this study, we used transformants harboring oncogenes that were amplified and were derived from a different species from the host cells. Thus, we suspect that poly(ADP-ribose) polymerase may have some mechanism to recognize species differences of DNA and/or amplified sequences. But we have no information on the problem of recognition of different species of DNAs because we have not yet examined the effect of BA on transfected mouse sequences in NIH 3T3 cells.

We tried to analyze the status of transfected sequences in NIH 3T3 cells by *in situ* hybridization, but this was not successful because of the instability of karyotypes of the NIH 3T3 cells. Thus, we do not know if the exogenous amplified sequences are present as double minutes, integrated into a chromosome, or as episomes.

To characterize the gene eliminating activity of poly(ADP-ribose) polymerase inhibitors, we focused attention on amplified sequences. We chose HL-60 cells, a hematopoietic cell line with amplified *c-myc* that is known to differentiate into granulocytes on treatment with poly(ADP-ribose) polymerase inhibitors. We also examined the effect of polymerase inhibitors on COLO 320HSR, which is an epithelial cell line in which the status of amplified *c-myc* has been determined (21). Thirdly, we used K562/ADM, a hematopoietic cell line in which a nononcogenic gene, *mdr-1*, is known to be amplified, but the effect of polymerase inhibitors on cell differentiation was unknown.

In the HL-60 cells used in this study, the *c-myc* gene was found not to be present as episomes, but probably as DMs or HSR (on chromosome 8). The *c-myc* gene was eliminated by treatment with poly(ADP-ribose) polymerase inhibitors. It is unknown, however, whether polymerase inhibitors eliminated all the *c-myc* genes in

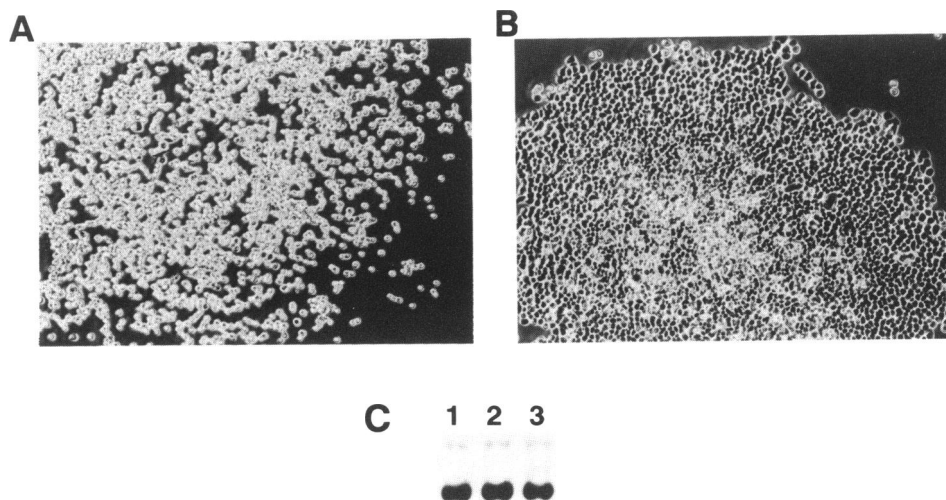


FIGURE 4. Effect of BA on COLO 320HSR. Inocula of 800 cells of a COLO 320HSR clone were cultured for 12 days in flasks coated with collagen type 1 with no addition (A) or with 5 mM BA (B). Nonadherent cells in the culture with 5 mM BA were separated from adherent cells by pipette. (C) Southern blot analysis was performed on *Xba*I digests of DNA from untreated cells (lane 1) and BA treated nonadherent (lane 2) and adherent (lane 3) cells with the *Pst*I-*Pst*I fragment of human *MYC* as probe.

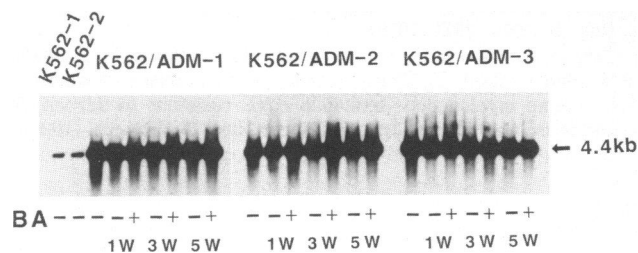


FIGURE 5. Effect of BA on the amplified *mdr-1* gene in K562/ADM cells. Clones K562/ADM-1, K562/ADM-2, and K562/ADM-3 were cultured in the presence of 5 mM BA for the indicated periods. Southern blot analysis was performed on *Hind*III digests of DNAs using a human *mdr-1* gene fragment, pMDR-1, as a probe.

differentiated cells or only reduced their copy number per cell. HL-60 cells differentiated into granulocytes and did not grow any more after granulocytic differentiation. Therefore, *in situ* hybridization will be necessary to determine whether the differentiated cells contain any copies of the *c-myc* gene. Gene loss was also observed on treatment with DMSO. The mechanism of induction of differentiation of HL-60 cells by DMSO has not been fully elucidated. As described in the text, DMSO at a concentration of 1.6% probably does not inhibit poly(ADP-ribose) polymerase. We are now measuring the amounts of poly(ADP-ribose) in HL-60 cells treated with BA or DMSO using an antibody against poly(ADP-ribose) to determine whether these compounds inhibit the poly(ADP-ribosylation) reaction *in vivo*. We are also examining whether other types of granulocytic inducers also cause loss of the *c-myc* gene.

We also used another cell line, COLO 320HSR, in which the amplified *c-myc* gene has been shown to be present as HSR on chromosome X. BA induced morphological changes of these cells but not *c-myc* gene loss. From these findings, two possibilities can be considered. One is that the *c-myc* gene in the HL-60 cells used in this study is present as DMs, and the sequence on DMs can be eliminated by poly(ADP-ribose) polymerase inhibitors. The other is that loss of the amplified endogenous *c-myc* is associated with granulocytic cell differentiation, which is associated with segmentation of the nuclei.

Another amplified endogenous gene, *mdr-1*, in K562/ADM, was not eliminated by poly(ADP-ribose) polymerase inhibitors. From these results we conclude that certain conditions are necessary for elimination of amplified endogenous genes by poly(ADP-ribose) polymerase inhibitors. We are now examining the effect of poly(ADP-ribose) polymerase inhibitors on integrated viral sequences to determine whether these exogenous sequences can be eliminated by poly(ADP-ribose) polymerase inhibitors.

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