Inhibition of carcinogen-induced cellular transformation of human fibroblasts by drugs that interact with the poly(ADP-ribose) polymerase system

Initial evidence for the development of transformation resistance

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Two types of interactions of 13 drugs with human fibroblasts were determined: (a) I_{50} of nuclear poly(ADP-ribose) polymerase, as assayed with isolated nuclei in vitro, and (b) the non-toxic concentration of drugs that prevented carcinogen-induced cell transformation of intact fibroblasts (RCF_1). In general, RCF_1 was much lower than I_{50}, and one antitransformer did not inhibit the enzyme in vitro, indicating that low-affinity enzyme inhibitory sites appear to play no role in the mechanism of prevention of cell transformation. Two enzyme inhibitors, caffeine and 1-methylnicotinamide, exhibited no antitransforming activity. Benzamide when applied in population doubling 1 induced resistance to cell transformation in population doubling 6 by carcinogens added at this stage.

Human fibroblast *Cell transformation* *Poly(ADP-ribose)polymerase*

1. INTRODUCTION

An inhibitor of poly(ADP-ribose) polymerase, benzamide [1], when present at non-toxic concentrations together with equally non-toxic quantities of ultimate carcinogens, prevents the induction of cell transformation in human fibroblasts that takes place in the absence of benzamide [2]. Similar observations were reported with C3H10T½ hamster embryo cells [3]. The antitransforming action of benzamide is confined to the early S phase and coincides with an increase of poly(ADP-ribose) polymerase activity [2], similar to the apparent induction of this enzyme in liver nuclei following benzamide feeding [4]. It would be expected that the multi-stage process of cellular transformation, leading to neoplasia, might be arrested by various agents at various stages (e.g., by prolonged exposure to protease inhibitors, cf. [5]) and the observed prevention of transformation by non-toxic concentrations of certain molecules that bind to the poly(ADP-ribose) polymerase system [2,3] is likely to indicate specific sites probably related to initiation. We show here that several in vitro inhibitors of poly(ADP-ribose) polymerase, at concentrations much below I_{50}, can function as antitransformers in intact cells. Conversely, some agents known to prevent carcinogenesis in animals can also inhibit poly(ADP-ribose) polymerase in vitro at relatively high concentrations. Therefore, the antitransforming propensity of molecules cannot be fully correlated with an inhibition of poly(ADP-ribose) polymerase and the cell biological effect of antitransformers is more probably reflected in high-affinity nuclear binding sites (RCF_1 as compared to I_{50}).

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2. EXPERIMENTAL

Preparation, culturing, synchronization, the method of exposure of human fibroblasts to carcinogens and to antitransforming drugs, and the assay technique for poly(ADP-ribose) polymerase, following quantitative removal of adsorbed drugs from the cell surface by both trypsin and antitrypsin treatment, have been reported [2,7]. Ultimate carcinogens employed were methylazoxymethanol acetate (MAMA, 7 μM) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, 0.7 μM) which were used interchangeably because at these non-toxic doses their transforming effects were indistinguishable. It is important to note that these low non-toxic concentrations of carcinogens are far below the doses many investigators use routinely in cell cultures to achieve responses in rates of poly ADP-ribosylations. For example, in a recent representative report MNNG was employed at nearly 50-times higher concentrations (cf. [8]) than in our studies. Toxicity, or rather its absence, was monitored by comparing cloning efficiencies in the presence and absence of drugs [2] and drug concentrations defined as RCF_1 (where relative cloning frequency is equal to one, meaning that cloning efficiencies with and without drugs are identical) were used. This criterion, as illustrated in fig.1 for benzamide, has been strictly employed for all drugs and drug combinations [2]. After exposure of cells in S of PD_1 (population doubling 1) to drugs, passages without drugs were continued for 20 PD and transformation frequencies were then determined by colony counts of transformed cells after transfer of cultures to a soft (0.33%) agar medium, representing anchorage-independent growth [2,7]. The dose response between the concentration of a typical antitransformer and the decrease in the number of colonies in soft agar (fig.1) portrays a sensitive quantitative measure of inhibition of cell transformation. For comparison of many drugs (table 1) only one concentration of antitransformer drug (i.e., RCF_1) is given, instead of a dose response curve which would involve almost unmanageable numbers of culture plates. For a total of 82 experimental series (table 1) between 1.6 and 2.0 $\times$ 10^6 transformed colonies were counted (one colony = a minimum of 50 cells) and the average rate of transformation by carcinogens was between 40 and 50 colonies per 10^4 cells [2].

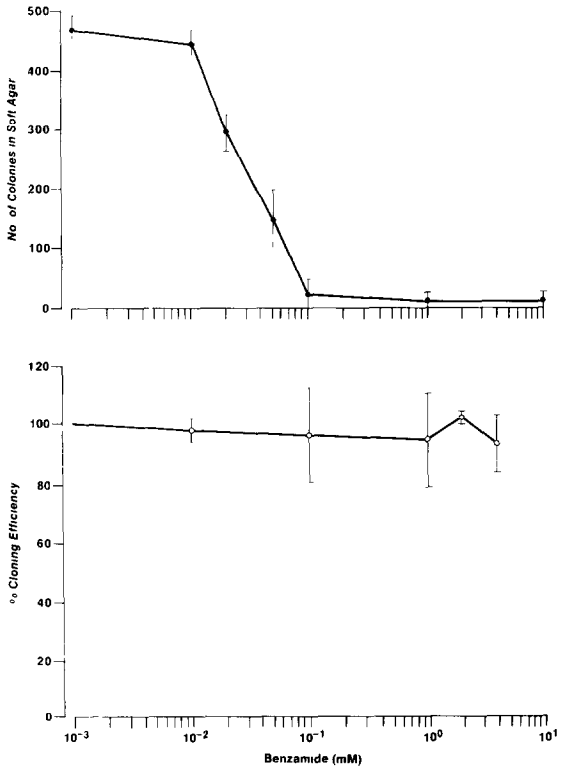

Fig.1. Correlation between the concentration of extracellular applied benzamide (between 0 and 1 mM, abscissa) and the number of transformed cell colonies capable of growing in soft agar (expressed as number of colonies/100000 cells; ordinate of upper curve). The transforming agent was 7 μM methylazoxymethanol acetate. The lower curve indicates the absence of cellular toxicity of either 7 μM methylazoxymethanol acetate (not shown separately) and varying concentrations of benzamide alone or in combination with the carcinogen. Ordinate of lower curve is % cloning frequency; eight parallel experiments were performed according to [2]: error bars represent SD, n = 8. RCF_1 is by definition between 0.1 and 1 mM benzamide.

Enzyme inhibitory indices (I_{50}) and the nature of inhibition were determined by standard kinetic models [9].

3. RESULTS AND DISCUSSION

As summarized in table 1, 10 out of 13 substances that at millimolar concentrations inhibit in vitro the nuclear poly(ADP-ribose) polymerase system, as determined by initial veloci-

Table 1

Prevention of carcinogen-induced cellular transformation of human fibroblasts by drugs that are inhibitory on poly(ADP-ribose) polymerase in vitro

No.	Experimental conditions	No. of transformed colonies per 5×10^4 cells	No. of experimental series	I_{50} (M)
1 (a)	0.7 μM MNNG or 7 μM MAMA	244.28 ± 13.6	21	–
(b)	a. + 1 mM BA	0	2	0.5×10^{-3}
(c)	1 mM BA	0	2	
2 (a)	+ 0.1 mM BHA	1.4 ± 0.6	2	
(c)	0.1 mM BHA	1.3 ± 0.3	2	5.0×10^{-3}
3 (a)	+ 0.1 mM Me-BHA	1.0 ± 0.4	3	
(c)	0.1 mM Me-BHA	0	3	20.0×10^{-3}
4 (a)	+ 0.7 μM NAL	2.0 ± 1.5	2	
(c)	0.7 μM NAL	0	2	2.0×10^{-3}
5 (a)	+ 0.8 μM NOV	8.0 ± 2.3	2	
(c)	0.8 μM NOV	0	2	10^{-3}
6 (a)	+ 0.2 μM LEV	6.0 ± 1.8	5	
(c)	0.2 μM LEV	0	5	10^{-3}
7 (a)	+ 69 μM COU	1.0 ± 0.7	5	
(c)	69 μM COU	13.0 ± 4	5	10^{-3}
8 (a)	+ 3 μM QU	2.8 ± 0.8	2	
(c)	3 μM QU	27.0 ± 3.3	2	0.25×10^{-3}
9 (a)	+ 0.4 μM ISO	0	2	
(c)	0.4 μM ISO	0	2	0.4×10^{-3}
10 (a)	+ 1 mM 1-Me-NA	220.0 ± 8.0	2 cf. [6]	
(c)	1 mM 1-Me-NA	2.0 ± 1.3	2	
11 (a)	+ 1 mM HMBA	4.0 ± 1.3	2	
(c)	1 mM HMBA	0	2 not inhibitory at 20 mM	
12 (a)	+ 1 mM CAFF	210.0 ± 15.0	2 cf. [6]	
(c)	1 mM CAFF	0	2	
13 (a)	0.8 μM PRIM	4.0 ± 1.5	2	2.0×10^{-3}
(c)	0.8 μM PRIM	10.0 ± 8.0	2	

BA, benzamide; BHA, butylated hydroxyanisole; Me-BHA, methyl ether of BHA; NAL, nalidixic acid; NOV, novobiocin; LEV, levimasole; COU, coumarin; QU, quercetin; ISO, isoquinoline; 1-Me-NA, 1-methylnicotinamide; HMBA, hexamethylene bisacetamide; CAFF, caffeine; PRIM, primycin. The cell biological effects of selected molecules were determined in synchronized human fibroblasts exactly as in [2]. Briefly, G_1 block was produced in freshly isolated and subcultured human fibroblasts (5×10^3 cell/cm^2) by nutritional deprivation (cf. [2]) then S induced by refeeding + insulin. Transforming agents and drugs were added in early S phase. The window of effectivity of both agents was the same as described (cf. [2]). Passages for 20 population doubling were continued and treated and control cultures seeded ($3–20 \times 10^6$ cells) into a semi-solid medium (0.33% agar, cf. [2]) to score for anchorage-independent colony growth (1 colony is defined as a minimum of 50 cells). The drug concentrations given in section 2 are RCF_1 (i.e., a non-toxic dose that inhibits transformation by 85–95%)

ty enzyme kinetics [9], proved to be potent antitransforming drugs. With the apparent exception of benzamide the concentrations of drugs sufficient to prevent nearly completely transformation are far below I_{50}. The anomalous behavior of benzamide is explained by its poor cellular penetration

(less than 1% of externally added drug appears in the nucleus, cf. [2]). Caffeine (no.12) and 1-methylnicotinamide (no.10) which are known inhibitors of the enzyme (cf. [6]) do not act as antitransformers, therefore in vitro kinetic effects alone are insufficient to predict an antitransforming property. The process leading to transformation inhibition takes about 10 h [2] whereas inhibition kinetics is determined within 1–2 min. It follows that differences in drug concentrations that cause enzyme inhibition or prevention of transformation suggest the participation of at least two types of binding sites and only the high-affinity site (RCF_1) is relevant to the prevention of transformation. Recent evidence shows the participation of a second nuclear binding site for benzamide which is localized at the coenzymic DNA of poly(ADP-ribose) polymerase (in preparation; and Proceedings of the VIIth International Symposium on ADP-ribosylations, Vitznau, Switzerland, Sept. 23–27, 1984) and we presume that other antitransformers also bind to this site. This question is the subject of further studies.

Benzamide (no.1) and its *ortho*- and *meta*-fluoro analogs, *m*-methoxybenzamide and the 2-(acetonyloxy),-2-(phenylacetonyloxy)-5-chlorobenzamides (not shown) are comparable antitransformers. Benzamides are toxic at 5 mM, except for the acetonyloxychloro derivatives which are nontoxic even at saturation. Butylated hydroxyanisole (no.2), a known inhibitor of carcinogenesis in animals [10] and its *o*-methyl derivative (no.3), prevent transformation in the cell culture system. The antibiotics, nalidixic acid (no.4) and novobiocin (no.5), at millimolar concentration, exert an inhibitory effect on eukaryotic topoisomerases [13,14] as well as on poly(ADP-ribose) polymerase (table 1), suggesting DNA-related binding sites. However, the antitransforming effect of these antibiotics (nos 4,5) and of the antibiotic primycin (no.13) occurs at much lower concentration than I_{50}, similar to levimasole, a drug (no.6) that is known to be supportive in cancer chemotherapy [11,12]. The bioflavonoid derivatives quercetin and coumarin (nos 7,8) are potent antitransformers at 3 and 69 μM, respectively. Quercetin has been shown to suppress tumor promotion [15] and contrary to previous reports is not a carcinogen [16], thus the marginal induction of transformed colonies by quercetin alone, that is abolished by the simultaneous presence of a potent carcinogen, may have little biological importance. The inhibitory effect of quercetin on tyrosine phosphokinase [17] and on other enzymes of the plasma membrane [18] requires 50–100-fold higher concentrations than sufficient for the prevention of carcinogen-induced phenotypic transformation (table 1, nos 7,8). Therefore, it seems improbable that these enzymes are significantly affected by quercetin when prevention of cell transformation takes place. Isoquinoline (no.9) bears structural homology to benzamide with respect to the position of the N atom relative to the benzene moiety.

Hexamethylene bisacetamide (no.11) is known to induce differentiation in erythroleukemia cells [19] simultaneously with an increase of poly(ADP-ribose) polymerase activity [20]. Its antitransforming effect in human fibroblasts suggests that the phenomenon of induced differentiation [19] and of induced resistance to transformation, both coinciding with an increase of poly ADP-ribosylation, may be related. Hexamethylene bisacetamide at 1 mM external concentration (RCF_1) almost completely prevented carcinogen-induced transformation, but even at 20 mM had no appreciable inhibitory effect on nuclear poly(ADP-ribose) polymerase. In the past [2] and in the majority of present experiments, prevention of transformation was demonstrated when both carcinogens and antitransforming drugs were present simultaneously

Table 2

Induction of resistance to cell transformation by benzamide

No.	Experimental conditions	No.of transformed colonies per 10^5 cells after PD_{20}
1	PD_1 0.7 μM MNNG alone	185 ± 18 (2)[a]
2	1. + 1 mM BA	5 ± 2 (2)
3	1 mM BA alone	0 (2)
4	1 mM BA at PD_1 and 0.7 μM MNNG at PD_6	0 (2)
5	0.7 μM MNNG alone at PD_6	130 ± 18 (2)

PD_1 and PD_6, population doubling 1 and 6, respectively; (2)[a], 2 experimental series; the cells used in these experiments exhibited greater resistance than the cultures shown in table 1, indicating biological variations. BA, benzamide

in early S phase. However, one exposure to benzamide alone at PD_1 confers resistance to transformation as demonstrated by the ineffectivity of the subsequent addition of carcinogens at PD_6, when traces of benzamide have long been removed by 6 serial passages involving exchanges of culture media (table 2). It is probable that benzamide is not unique in producing resistance to transformation. The mechanism of this phenomenon is subject to further studies. The antitransforming effect of certain drugs (table 1) depends strictly on their low concentration that must be at non-toxic levels. Raising the drug concentration to I_{50} will not only abolish the antitransforming effect but also produce cell toxicity and can reinforce carcinogens (cf. [2]).

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