Biochemical Pharmacology, Vol. 28, pp. 2723–2728

0006-2952/79/0915–2723 $02.00/0

ENHANCED UPTAKE OF ACTINOMYCIN D IN CULTURED MAMMALIAN CELLS BY THE ANTI-INFLAMMATORY, NON-STEROID DRUG BENZYDAMINE

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(*Received* 2 *August* 1978; *accepted* 26 *March* 1979)

Abstract—The anti-inflammatory, non-steroid drug benzydamine, used at non-toxic concentrations ranging from 10 to 50 $\mu g\ ml^{-1}$, markedly enhanced the uptake of the antiblastic drug actinomycin D in several lines of cultured mammalian cells including tumor cells. In particular, the synthesis of RNA of cells derived from African Green Monkey Kidney, which showed a degree of resistance to actinomycin D, was rendered sensitive to this drug by benzydamine. The nucleocytoplasmic distribution of actinomycin D at equilibrium was not altered by benzydamine, nor did this drug increase the amount of actinomycin D taken up by isolated nuclei. Experiments performed with radiolabeled benzydamine showed that the binding of the drug to the cell is rapid but promptly reversible, almost all bound material sedimenting at 100,000g in the ultracentrifuge. The experimental evidence favours the idea that benzydamine exerts the described effects by interacting with plasmamembrane and allowing for a more effective penetration of actinomycin D into the cell.

Actinomycin D (AMD), like other amphiphilic drugs, seems to penetrate the sensitive cells by a simple diffusional process through the plasmamembrane, the rate of which depends on the fluidity of the lipid bylayer as well as on extra-lipid factors which influence such fluidity [1]. In evaluating the degree of inhibition of transcription by AMD and related drugs, it is quite difficult to distinguish variations in the actual inhibition of RNA synthesis from changes in the rate of drug penetration into the cell. This uncertainty arises from the high number of AMD-binding sites and their marked avidity [2].

An approach to overcome this difficulty is to evaluate the effects of AMD in presence of compounds acting on cell membrane. For instance, Medoff *et al* [3], showed that amphotericin B, an antibiotic which, by binding to steroids, increases membrane permeability [4], permits the entry of AMD in AMD-resistant cell lines which do not normally take up the drug to any significant extent [5]. Similar results were obtained by treating resistant cells with the non-ionic detergent Tween 80 [6], even though it cannot be excluded that the detergent may enter the cell and modify the affinity of the nuclear target for AMD [2].

During our studies on the mechanism of the natural resistance of the African Green Monkey Kidney (AGMK) cell lines to AMD [7, 8] we found that the anti-inflammatory, non-steroid drug benzydamine (BD), used at non-toxic concentrations, restored the sensitivity of these cells to AMD by increasing the uptake of the drug. The mechanism suggested for this effect is an increased AMD entry following the interaction of BD with the cytoplasmic membrane.

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MATERIALS AND METHODS

Cell cultures. 37 RC, CV1 and Vero, all epithelial-like cultures derived from African Green Monkey Kidney (AGMK) cells, and HeLa, mouse L strains and Erlich ascite tumor cells were used throughout this study. All cultures were routinely assayed for mycoplasma contamination by the method of Barile and Schlimke [9] as well as by electron microscopic observations. Apart from Erlich ascite cells which were grown in infected mice [2], all other cells were grown as monolayers in Falcon bottles (flow area : 25 cm^2) with Eagle's medium (MEM) supplemented with non-essential aminoacids, 5% fetal calf serum and penicillin, streptomycin and kanamycin, 50, 100 and 10 $\mu g ml^{-1}$, respectively. The kinetics of AMD uptake and efflux and uridine incorporation into either TCA-precipitable or whole cell materials was carried out using cells either grown in Falcon bottles or transfered into Linbro plates each containing 24 small wells (surface area: 1.8cm^2) and seeded at a density of $1.10^5 cm^{-2}$ in various MEM volumes, as indicated in single experiments. Unless otherwise stated, all experiments were performed 24 hr after seeding at a cell density of $2 \cdot 10^5$ cells/cm^2.

Uridine incorporation into acid-precipitable material. Serum-free MEM containing 10 $\mu Ci ml^{-1}$ of [^{3}H]uridine (sp. act. 11 $Cimmole^{-1}$) was added to the confluent monolayers (either untreated or treated with various amounts of AMD and/or BD) in Linbro plates. After a 30 min pulse, the incorporation was stopped by washing with cold phosphate buffered saline (PBS) and the monolayers were dissolved with sodium dodecyl sulfate-EDTA-papain buffer (SDS-buffer) [8, 10] containing NaCl 10mM, Tris–HCl 10mM,$Mgcl_2$ 1mM, EDTA 10 mM, papain 20 $\mu g\ ml^{-1}$, cystein 20 $\mu g\ ml^{-1}$ and SDS 0.5% (w/v), pH 7.0. Aliquots of the lysate were precipitated onto 0.5μm Millipore filters (Millipore Corp., Cat. No. HAW P304FO) by adding trich-

loracetic acid (TCA) to a final concentration of 10% (w/v). The filters were washed with 5% TCA added with 0.5% SDS, dissolved in Bray's solution and counted for incorporated radioactivity (see below).

Uptake and efflux of AMD. (i) *Whole cells.* Confluent monolayers in Linbro or in Falcon bottles were exposed to [^{3}H]AMD (5 μCi ml^{-1}; sp. act. 6.3 Ci mmole^{-1}) and unlabeled drug up to a concentration of 5 μg ml^{-1}. At different time intervals, monolayers were carefully washed with PBS, dissolved in SDS buffer and counted for total incorporated radioactivity. The endocellular concentration of AMD was calculated on the basis of previous assumptions [10]. To determine the efflux of AMD, cells were loaded for 1 hour with [^{3}H]AMD (as described above), washed with cold MEM containing unlabeled AMD (5 μg ml^{-1}) and then added with various volumes of prewarmed MEM. At different time intervals, the cellular radioactivity was counted. The initial rate of uptake, called 'influx', was measured by stopping the incubation after 100 sec.

(ii) *Isolated nuclei.* Ghost monolayers in Falcon bottles were prepared by the method of Tsai and Green [11], as modified by Rovera, Mehta and Moul [12]. After gentle washing with MEM, cells were exposed to [^{3}H]AMD (see above), in presence or absence of BD, and the amount of AMD taken up measured as reported elsewhere [10].

(iii) *Nucleocytoplasmic fractions.* Monolayers in Falcon bottles were exposed to radioactive AMD, in the presence or absence of BD. At different time intervals, cells were washed with cold MEM and separated into nuclei and cytoplasmic fractions as described by Penman [13] and the radioactivity measured.

Binding of BD to whole cells and nucleocytoplasmic fractions. [^{14}C]BD (sp. act. 26.55×10^6 c.p.m. mg^{-1}) and unlabeled BD to a concentration of 30 μg ml^{-1} in MEM was given to monolayers in Falcon bottles. At different time intervals, monolayers were washed with cold MEM containing 30 μg ml^{-1} of cold BD. Nuclei and cytoplasmic fractions were separated as described above and radioactivity counted. In BD-elution experiments, cells were loaded for 30 min with radioactive BD, then quickly washed off with BD-free MEM and left to elute the drug for 10 min. The degree of elution was measured by radioactivity determination of whole cell materials.

Implantation assay. Cells were suspended in MEM at a density of 2.0×10^5 ml^{-1} in a total volume of 5 ml (unless otherwise specified). Just before cell seeding (zero time) as well as at different time intervals during incubation, aliquots of medium (0.1 ml) were counted for number of cells in a Neubauer hemocytometer chamber.

Radioactivity determinations. The radioactivity was determined in Bray's solution with a Beckman liquid scintillation counter. Counting efficiency was estimated by the two channels ratio using the external standard method.

Chemicals. All radioactive precursors were obtained from Amersham Radiochemical Center, England. Unlabeled AMD was given by Serva, Heidelberg, West Germany. Both radioactive and unlabeled benzydamine (1 benzyl-3 [3-dimethylaminopropoxy] 1 H indazole-HCl) were a kind gift of Angelini, Ancona, Italy.

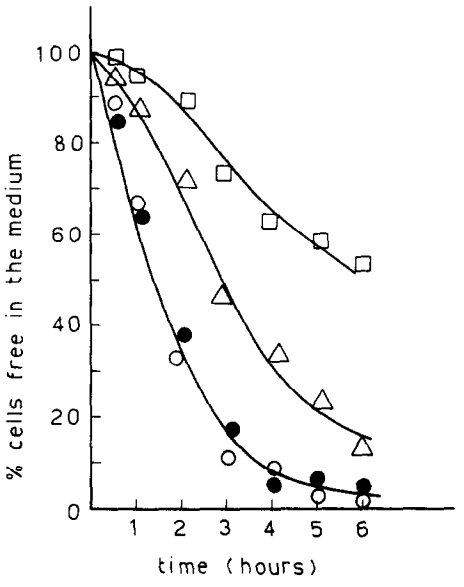

Fig. 1. The effect of BD on implantation of 3T7 RC cells to the plastic surface. Cells were seeded at density of 2.0×10^5 ml^{-1} in the presence of the desired amount of BD (30 μg ml^{-1} ●—●; 50 μg ml^{-1} △—△; 100 μg ml^{-1} □—□) or in absence of BD (○—○). At different time intervals, the percentage of cells free in the medium was counted. For other details, see text.

RESULTS

1. The effect of benzydamine on cell viability, adhesiveness and implantation. In order to evaluate the toxicity of BD for cultured mammalian cells, the effect of the drug on typical morphofunctional parameters of the cultures was determined, either by treating confluent monolayers or by treating the cells during their implantation stage. No cell detachment from dish was

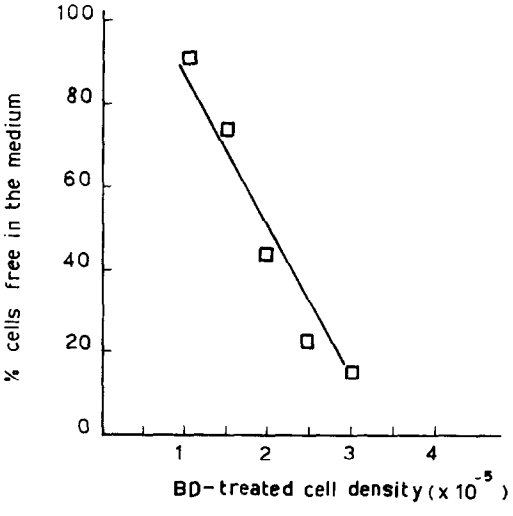

Fig. 2. The relationship between BD activity on implantation and cell density. Cells at the indicated density in a total volume of 10 ml were treated with BD (100 μg ml^{-1}) and the percentage of unimplanted cells was recorded after 5 hr from seeding. For other details, see text.

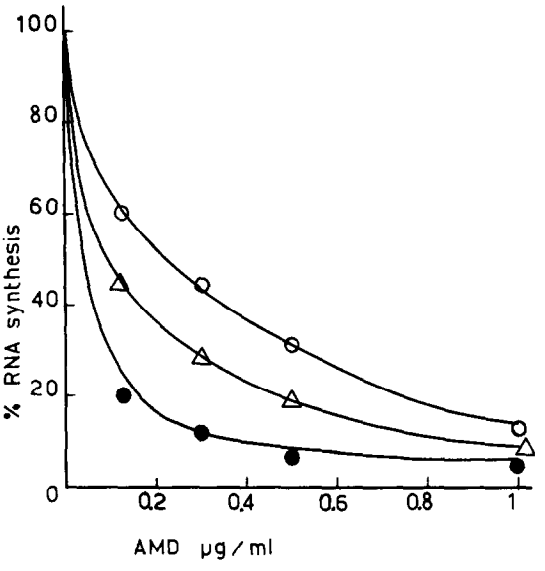

Fig. 3. The effect of BD on inhibition of RNA synthesis by AMD. 37 RC monolayers in Linbro plates were incubated in the presence of the indicated concentrations of AMD and BD (10 μg ml^{-1} △—△; 30 μg ml^{-1} ●—●). The control was monolayers treated with AMD only (○—○). After 10 min exposure to the drugs, cultures were added with [^{3}H]uridine (10 μCi ml^{-1}) and the incorporation into acid-precipitable material after 30 min of incubation was measured. RNA synthesis was expressed by percentage of a control treated with neither AMD nor BD.

observed up to a BD concentration of 80–100 μg ml^{-1}. At higher concentrations, cells first rounded up then detached in a number proportional to the dose of BD employed. The addition of BD at the implantation stage (cells trypsinized and resuspended in BD-containing medium) remarkably delayed or excluded the implantation, here too in a dose-dependent way (Fig. 1). The effect was, however, not remarkable up to 30 μg ml^{-1} of drug.

Unattached cells held in presence of BD for a maximum of 6 hr resumed a rapid implantation rate as soon as they are eluted free off the drug showing that toxic effect was readily reversible. Apart from the dose, the effect of BD on cell implantation depended in a strict proportional way on the number of cells matched with the drug (Fig.2). Similar results were obtained if cell viability was tested by the dye exclusion method.

Results were also comparable in all cell lines examined and therefore are reported here only for 37 RC cells (Figs. 1 and 2].

2. Effect of benzydamine on transcription and on its sensitivity to actinomycin D. Up to a concentration of 50 μg ml^{-1}, BD was shown not to affect the rate of uridine incorporation into acid-insoluble material, in any cell line examined. Nor did it seem to affect the rate of precursor uptake into the cell as measured in experiments of uridine incorporation at early times (2–5 min). Similarly, BD did not affect the uptake of either glucose or aminoacids.

On the other hand, the exposure of cells to BD (10 to 30 μg ml^{-1}) significantly increased the degree of inhibition of RNA synthesis brought about by actinomycin D (AMD) (Fig. 3). This effect was mainly appreciated using low, sub-inhibitory doses of AMD. From dose-effect curves, like that shown in Fig 3, it can be seen that 50 μg ml^{-1} BD is equivalent, as far as the rate of the inhibition of transcription is concerned, to a ten-fold increase in AMD concentration.

3. Uptake and elution of actinomycin D in presence of benzydamine. To get insight into the mechanism by which BD enhanced the sensitivity of the transcription of cultured cells to AMD, we measured the rate of uptake and elution of AMD in presence of BD. In all

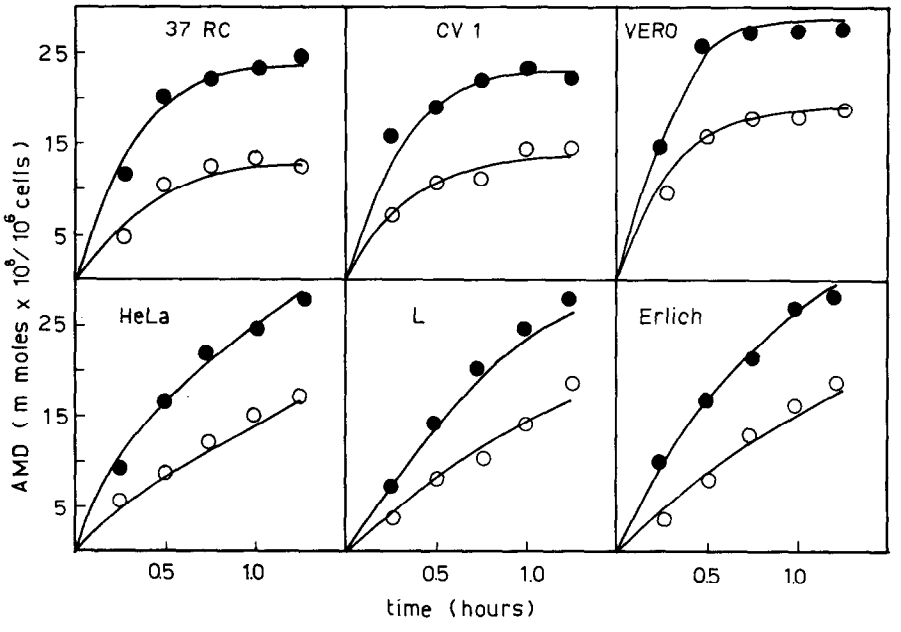

Fig. 4. Effect of BD on AMD uptake in various cell cultures. Treated with BD 30 μg ml^{-1} (●—●) and untreated (○—○) cultures. For details, see text.

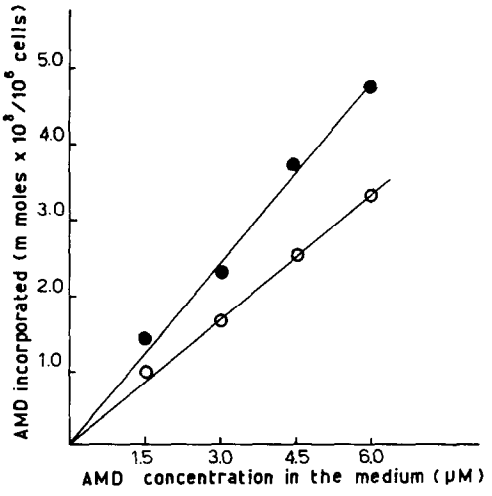

Fig. 5. The effect of BD on the initial rate ('influx') of AMD uptake in 37 RC cells. 37 RC cultures in Linbro plates were exposed to MEM containing [^{3}H]AMD at the indicated concentrations in the absence (○—○) or in the presence of 30 μg ml^{-1} BD (●—●) After 100 sec, the amount of intracellular AMD was measured as described in Materials and Methods.

cell lines examined we found that BD, at the usual concentrations, clearly stimulated an increase in the rate of AMD uptake, mostly when cells were shortly (15 min) pretreated with BD (Fig. 4). For each cell culture, however, the overall shape of AMD-uptake curve (including the equilibrium plateau, when present) was the same regardless of the presence of BD.

In AGMK cell lines, AMD uptake reached its equilibrium from 60 to 90 min and the equilibrium concentrations in the presence of BD were generally between 1.5–2.0 times greater than in absence of BD.

Table 1. Elution of AMD* in different volumes of effluent medium, in presence or absence of BD†.

Effluent volume (ml)	Control		BD-treated	
	10 min	30 min	10 min	30 min
0.1	72	64	87	82
2.0	76	53	81	54

* Expressed as percentage of zero time value. Single figures are the mean of three independent experiments.
† 30 μg ml^{-1}.
For other details, see Materials and Methods.

The uptake of AMD in other cell lines (HeLa, L, Erlich all of neoplastic nature) did not reach its equilibrium at 90 min, but later on, and the overall kinetics of the uptake was different from that of AGMK cells; nevertheless, BD provoked a comparable increase in AMD uptake (Fig. 4).

On the other hand, the uptake curve is the sum of the influx and the binding of the drug. To distinguish the two effects we measured the initial rate (100 sec) of AMD uptake, and called it 'influx' assuming that, at such a very early time, the amount of intracellular AMD is a reflection of drug permeation rather than binding. Figure 5 shows that, in a range of AMD external concentrations, BD did always significantly increase AMD influx in 37 RC cells, as well as in other cell lines (not shown).

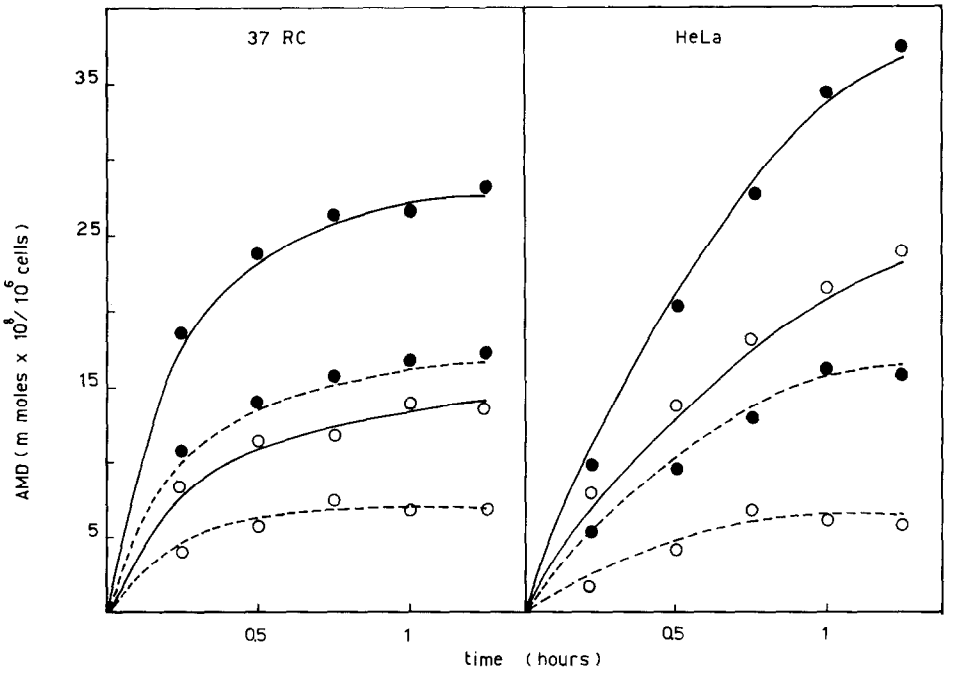

Fig. 6. The distribution of AMD over nuclei (continuous line) and cytoplasmic fraction (dashed line) in 37 RC and HeLa cells in the presence (●—●) or absence (○—○) of BD (30 μg ml^{-1}). For other details, see text.

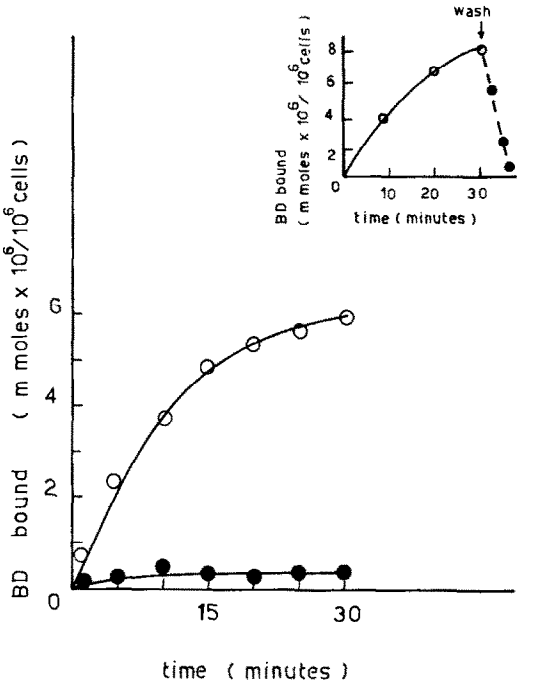

Fig. 7. Binding of BD to nuclear (●—●) and cytoplasmic (○—○) fractions of 37 RC cells, as described in Materials and Methods. The inset shows the elution of BD after 30 min loading with the drug and washing with BD-free medium. For other details, see text.

The effect of BD on elution rate of AMD was also investigated. It was of major interest to study this effect in AGMK cells which are known for their rapid elution of actinomycin D [2] whereas other cells, like HeLa and Erlich have a very low rate of AMD elution [2, 10]. This difference has been related to the capacity of AGMK cells of early recovering RNA synthesis [7, 10]

It was seen that the effect of BD on AMD elution from 37 RC cells loaded with AMD at equilibrium critically depends on the volume of efflux medium. As shown in Table 1, elution of AMD was significantly decreased by BD (mainly evident at 30 min) only when efflux medium ranged from 0.1 to 1.0 ml. A 20 times change in this volume (from 0.1 to 2.0 ml) drastically abolished any effect of BD on AMD elution. Altogether, the data reported above suggest that BD, while stimulating AMD influx, does not affect AMD efflux even though larger elution volumes should be used to definitely establish this point.

4. On the mechanism of increased actinomycin D uptake by benzidamine. To try to elucidate the mechanism(s) by which BD enhanced AMD uptake in cultured cell lines, several experiments were performed. First of all we studied whether BD altered the nucleocytoplasmic distribution of AMD. Both in 37 RC and in HeLa cell lines the amount of AMD taken up in presence of BD was distributed over nuclei and cytoplasm in the same ratio as that normally taken up in absence of BD. Thus, there was no relative accumulation of AMD in the nucleus as expected if BD modified the binding sites for AMD (Fig. 6). If isolated nuclei were exposed to BD no increase in AMD uptake could be observed (Table 2). Finally, pretreatment of cells with BD at concentrations capable to increase AMD uptake, did not so modify cell nuclei as to determine an enhanced drug uptake following their isolation from cells.

Table 2. The effect of BD on AMD uptake* in whole cells and isolated nuclei

	AMD (moles × $10^8/10^6$ cells)†	
	− BD	+ BD‡
Cells	9.5	20.4
Nuclei	25.0	24.4

* Calculated after 30 min exposure to 5 μCi ml^{-1} of [^{3}H]AMD.

† Or nuclei isolated from a correspondent number of cells.

‡ At a concentration of 30 μg ml^{-1}.

All these experiments support the conclusion the BD-induced, increased AMD uptake is not due to a relative increased binding of AMD to nuclear chromatin, for either unmasking of new, or increased affinity of the usual, binding sites. On the contrary, it seems to depend on an accelerated permeation of AMD through the plasmamembrane.

If this were so, plasmamembrane itself would be expected to be a major target of BD action. Experiments performed with radiolabeled BD (see Materials and Methods) have shown that this drug rapidly associates with cell approaching equilibrium after 30 min exposure (Fig. 7; only very small and time-constant amounts of drug were associated with nuclei). Almost all the radioactivity was detected in the cytoplasmic fraction and was precipitable at 100,000 g. The radioactivity was also rapidly eluted following BD withdrawal from medium (Fig. 7, inset). This seems consistent with the idea that BD binds to plasmamembrane and possibly also the intracytoplasmic membranes.

The low specific activity of radiolabeled BD precluded more selective studies on binding of BD to cell membranes.

DISCUSSION

Cultured cell lines derived from African Green Monkey Kidney (AGMK) like Vero, 37 RC, CV1 and others show a degree of resistance to AMD, as compared to all other cell lines, expressed by both a lower cytotoxicity [14] and rapid recovery of RNA synthesis after maximal AMD suppression [7, 8]. The reasons for this natural resistance are not perfectly known but it has clearly been shown that AGMK lines, in contrast to AMD-sensitive lines like HeLa and Erlich, retain a lower amount of endonuclear AMD at equilibrium and elute the drug at a much higher rate [2, 10]. The situation looks quite similar to that reported by Williams and McPherson [15] for virus-transformed cells which are more resistant to AMD than their normal counterparts. There are also genetically-selected cell lines which are completely refractory to AMD and other amphiphilic drugs as they completely exclude the entry of drug into the cell [16, 17].

We have shown here that BD, an analgesic, anti-inflammatory, non-steroid drug induced a marked increase of AMD uptake in all cell lines examined. As a

consequence, the endocellular amount of AMD is greater and the inhibition of RNA synthesis stronger than in absence of BD. Since the initial uptake rate (at 100 sec, 'influx') of AMD is enhanced by BD, we suggest that BD may act by facilitating AMD entry into the cell. All experimental results support this view. There is no evidence for BD exerting any effect on AMD efflux. It is important to point out that BD increases AMD uptake also in those cell lines like HeLa and Erlich, which do not have an appreciable AMD-elution component [2, 10].

Despite a number of studies on the anti-inflammatory activity of BD [18,–20] the mechanism of action of this drug is not known. Our experiments on cellular binding of radioactive BD showed that the intact cell binds the drug effectively but in a strong reversible way suggesting that non-covalent bonds are involved. For instance, once the equilibrium has been reached (at approx. 30 min), about one third of the total BD (30 μg) is bound by 10^6 cells and almost all this quantity bound is released during the first 5 min of elution. After cell breakage, the radioactivity of [^{14}C]BD sediments with the 100,000g fraction of the cytoplasm. Moreover, isolated nuclei were shown not to bind a significant amount of BD and, coherently, BD-treated, isolated nuclei did not take up more AMD than the untreated ones. Finally, the nucleocytoplasmic distribution of AMD in BD-treated cells exactly parallels that measured in control cells.

From all the data reported above it seems unlikely that the BD-induced enhancement of AMD uptake may be due to any unmasking, or increase in affinity, of AMD-binding sites in the chromatin. Indeed, we suggest the plasmamembrane as the major site of the BD effect so that changes in this membrane may allow for a greater penetration of AMD. However, our results do not exclude the possibility that other, intracytoplasmic membranes may bind BD and indirectly influence the uptake of AMD. In view of what has been reported by Biedler and Riehm [17], it is possible that other amphiphilic drugs, which penetrate the plasmamembrane by the same route as AMD, are also allowed to enter the cell more efficiently by BD. It is of interest to note, however, that the ability to take up the small metabolic precursors like glucose or aminoacids is not influenced by BD, showing that transport systems may not be affected by BD, and that the drug does not provoke a generalized membrane permeabilization like amphotericin B [4].

The above conclusion is also in line with the effect of BD on cell adhesiveness which is a surface-associated property. That the plasmamembrane is a site of BD activity is also supported by the observation that BD and other anti-inflammatory non-steroid drugs markedly influence the hemolysis of red blood cells by heat or hypotonic solutions [19].

This study gives additional evidence that the rate of AMD-permeation is a critical factor in modulating the inhibition of transcription. This particularly applies to those cellular systems like AGMK lines and virus-transformed cells where a major part of AMD, though essential for the inhibition of transcription, is loosely bound to the chromatin and can rapidly be eluted from cells determining prompt recovery of the transcription [10].

In principle, BD could be of assistance in treating tumor cells *in vivo* which are resistant to AMD and other antiblastic drugs, provided sufficient amounts of BD can be attained *in vivo* to this purpose. BD would also make therapeutically effective lower doses of AMD and prolong the beneficial effects of AMD even during decline of serum or extracellular drug level. It should be considered, however, that BD would concomitantly increase the uptake of AMD in other cells of the organism, possibly enhancing toxic effects. This consideration also applies to the Amphotericin B-induced permeabilization of AMD-resistant tumor cells, as reported by Medoff *et al* [3].

Acknowledgements—This research was in part supported by a research grant from the National Research Council, Italy) (Progetto Finalizzato Virus) to A. Benedetto. The Authors gratefully acknowledge the technical assistance of Mr. E. Illomei and Mr. C. Ianniciello.

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