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The antimitotic drug 4,6-dimethyl-2-amino-3,4,5-trimethoxyphenyl-pyrimidine inhibits the nucleoside transport system of cells from various animal species

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Abstract. 4,6-dimethyl-2-amino-3,4,5-trimethoxyphenyl-pyrimidine (B-31) is a pyrimidine derivative previously reported to arrest the mitotic cycle of mammalian cells in metaphase. In the present study it is shown that B-31 also acts as a specific inhibitor of the cellular uptake of nucleosides. The uptake of purinic and pyrimidinic nucleosides is inhibited by 80–90% at concentrations in the range 5–20 μg/ml, whereas those of nucleobases, leucine, choline and glucose are unaltered at the maximum nontoxic dose of B-31 (25 μg/ml). Various mammalian (human, monkey and murine) and avian cell are equally sensitive to the inhibition of nucleoside transport. The antimitotic effect of B-31, by contrast, is species-specific: human cells are the most sensitive whereas monkey and chicken fibroblasts appear resistant to this effect. Both effects can be reversed by removal of B-31 from the medium; inhibition of nucleoside transport can also be reversed by high doses of the nucleosides themselves.
Key words. Antimitotic drug; nucleoside transport inhibitors.

4,6-dimethyl-2-amino-3,4,5-trimethoxyphenyl-pyrimidine (B-31) is a pyrimidine derivative shown to arrest a high proportion of mammalian cells in metaphase, both in vitro and in vivo[10]. In both cases, however, this substance was far less potent than the known antimitotic agents colchicine and vinblastine, used as reference compounds. Thus the lowest dose of B-31 capable of producing the maximum number of metaphase-arrested HEp2 cells (70–80%) was 34 μM (10 μg/ml), compared with 0.02 μM (0.01 μg/ml) of colchicine and 0.001 μM (0.001 μg/ml) of vinblastine. The mitostatic effect of B-31 on the bonemarrow cells of Swiss mice was less pronounced than that observed in vitro and was produced by a dose (1000 mg/ml) 100 times higher than the most effective concentration of vinblastine. However, the cytotoxicity of B-31 was consistently lower than that of colchicine and vinblastine, and its mitostatic activity appeared more easily reversible than that of vinblastine. Even though these results excluded the potential use of B-31 as an antitumor agent, they indicated that it might be a useful tool for cell synchronization. In the course of a study aimed at the characterization of the effects of B-31 on cell multiplication and metabolism, a strong inhibitory effect on the transport of nucleosides across the cell membrane was detected.

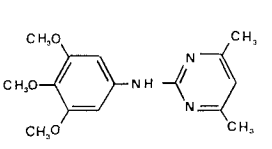
Nucleoside transport could be inhibited in cells from various animal species; by contrast, the antimitotic effect appeared species-specific. A possible correlation between the two effects will be discussed.

Materials and methods

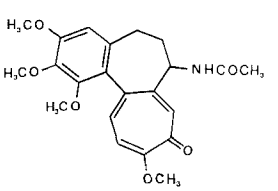
Materials. B-31 manufactured for experimental purposes (Istituto Chemioterapico Italiano, Milan) was used in these experiments. Colcemide (demecolcin, deacetyl-*N* methyl colchicine), nucleosides and the other compounds used in this study were all from Sigma (Milan). Radioactive compounds were from Amersham Laboratories (Milan).

Cells and culture conditions. HEp2 and HeLa (human), Vero (monkey), and L929 (mouse) cell lines were purchased from ATCC (USA). Chick embryo fibroblast primary cultures (CEF) were obtained by trypsinizing 11 day embryos. All these cultures were grown in Eagle's minimum essential medium (MEM) supplemented with 7% decomplemented foetal calf serum (FCS). Human peripheral blood lymphocyte cultures (PBMC) obtained from heparinized venous blood[5], were cultured in RPMI 1640 containing 20% FCS and stimulated with PHA for 36 h before use. Experiments involving asynchronous populations were performed on cells growing exponentially for at least 3 cell cycles. Cell counts were made with a ZM Coulter counter and cell viability was determined by the trypan blue dye exclusion method.

Determination of metaphase-arrested cells. Numbers of metaphase-arrested cells were determined as follows: cell cultures were first exposed to a hypotonic solution (0.7 M KCl; 0.01 M phosphate buffer pH 7.2) and then to a methanol-acetic acid (3:1) fixative solution. The mitotic index was calculated from 500 cells/sample in



B-31



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random fields which showed no striking differences in the numbers of interphase nuclei and of cells in metaphase. Details of individual experiments are given in the legends to figures and tables.

Results

Effect on cell growth. In the presence of 50–100 µg/ml of B-31, the number of viable HEp2 cells decreased with increasing time of treatment, indicating cytoxicity at these concentrations. In cultures exposed to lower doses (12–25 µg/ml) the initial number of cells remained constant for up to 3 days of continuous treatment (fig. 1); and, upon removal of the medium containing B-31, the cells started to grow at the rate of untreated controls, indicating a fully reversible cytostatic effect (fig. 2).

Effect on macromolecular synthesis and cell division. The amount of tritiated thymidine and uridine incorporated into nucleic acids of cultures treated with B-31 was progressively reduced by increasing drug concentrations (fig. 3). Within 30 min of the addition of the maximum mitostatic non-cytotoxic dose (25 µg/ml), the uptake of thymidine and uridine was 20 and 30% of untreated controls; 1.5 µg/ml was still sufficient to reduce the uridine incorporated into RNA by 50% (fig. 3A). Inhibition of uptake of uridine and thymidine was promptly reversed upon removal of the compound (not shown). The uptake of leucine, unchanged even at the maximum dose tested during the 30 min of treatment (fig. 3A), was impaired in a dose-dependent fashion after 24 h of continuous exposure to B-31 (fig. 3B). Duplicate cultures used to count cells arrested in metaphase showed increasing numbers of mitoses at doses of B-31 up to 25 µg/ml (fig. 3C).

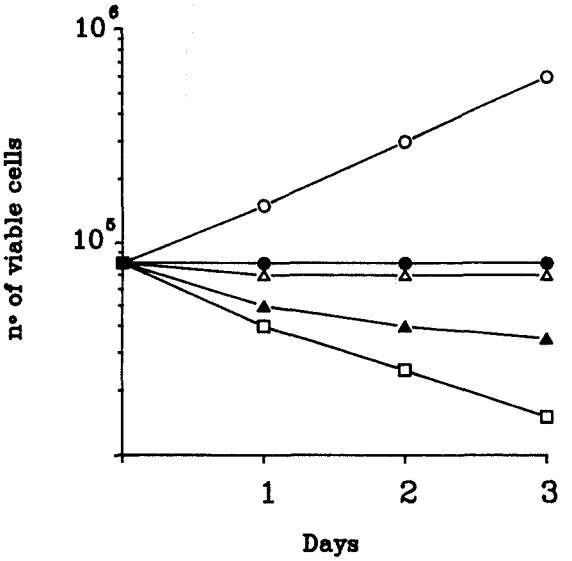

Figure 1. Effect of B-31 on cell growth. Duplicate cultures of HEp2 cells (8×10^4 cells/well) were incubated without (—○—) or with B-31 at the following concentrations: 100 µg/ml (—□—); 50 µg/ml (—▲—); 25 µg/ml (—△—); 12 µg/ml (—●—). Number of viable cells was determined at 24 h intervals.

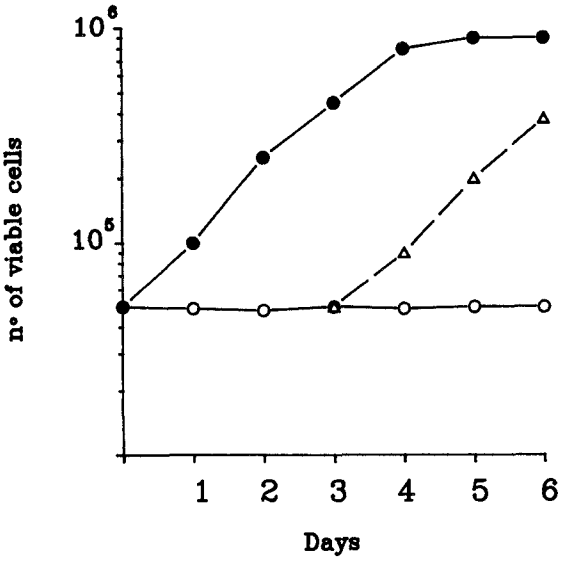

Figure 2. Reversibility of the inhibitory effect of B-31 on cell growth. Duplicate cultures of HEp2 cells (5×10^4 cells/well) were incubated without (—●—) or with (—○—) 12 µg/ml of B-31. After 3 d, B-31 containing medium was removed from a set of the cultures (—△—). The number of viable cells was determined at 24 h intervals.

Effect on the DNA synthesis of synchronous cells. In synchronized cells, the uptake of thymidine during the S phase was reduced by B-31 to 30% of that of controls (fig. 4), whereas that of uridine, followed throughout G1, S and G2 phase, was always approximately 6% of control values (data not shown). However, synchronized cells continuously exposed to B-31 were able to progress through the following stages up to mitosis, where more than 90% of cells were arrested in metaphase.

Effect on nucleoside transport. The interference of B-31 with the nucleoside transport system suggested by these results was confirmed by the data reported in table 1 and figure 5. At a temperature (16 °C) that inhibited enzymatic reactions during the pulse, B-31 prevented the incorporation of uridine into RNA provided that the compound was included in the labeling medium, whether or not present during the chase at 37 °C. If, on the contrary, B-31 was added when the labeled precursor had already entered the cell, the TCA-insoluble radioactivity recovered was identical to that of untreated controls (table 1). Secondly BVDU**, a well known nucleoside analogue endowed with selective antiviral activity against HSV-1, did not inhibit the replication of this virus when tested in the presence of B-31 (table 2). B-31 itself was tested for antiviral activity against examples of DNA and RNA viruses, including HIV-1, but in no instance was there any significant antiviral activity (data now shown).

Effect on the uptake of various labeled precursors and reversal of inhibition of nucleoside transport. The inhibitory effect of B-31 on the cellular uptake appeared

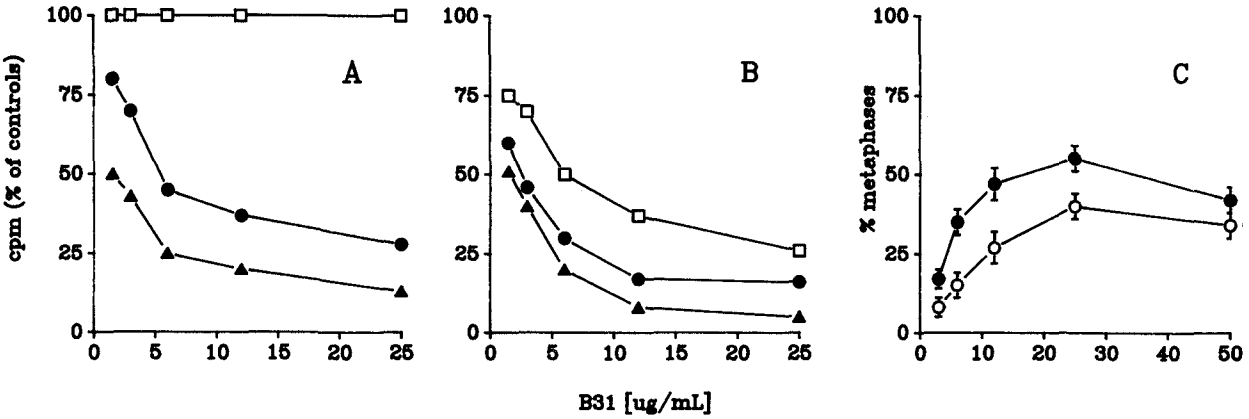

Figure 3. Effect of B-31 on macromolecular synthesis and cell division. Duplicate cultures of exponentially growing HEp2 cells (3×10^5 cells/well) were incubated with or without the indicated concentrations of B-31. Immediately after the addition of the drug (graph A) or after 22 h (graph B), the cultures were pulse-labeled for 30 min with 3H-leucine (2 μCi/ml; 5 × 10-4M) (—□—); 3H-TdR (2 μCi/ml; 10-7M) (—●—); 3H-UR (2 μCi/ml; 10-7M) (—▲—). TCA-insoluble radioactivity was determined in a Beckman scintillation counter. Percentages of cells arrested in metaphase (graph C) were counted after 12 (—○—) and 24 (—●—) hours (see 'Materials and methods').

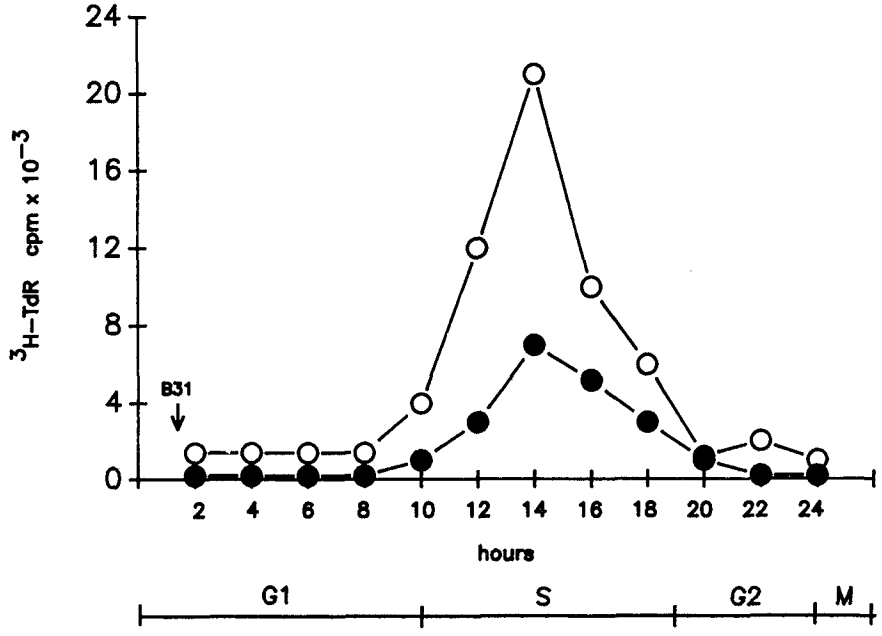

Figure 4. Effect of B-31 on thymidine uptake of synchronous cells. HEp2 cells were synchronized in the M/G1 phase of the cell cycle by collecting and plating mitotic cells after treatment with 0.02 μg/ml of colcemide. 20 μg/ml B-31 was added immediately after the completion of mitosis. The incorporation of 3H-TdR into TCA-insoluble material in untreated (—○—) and B-31 treated (—●—) cells was determined during a complete cell cycle (24 h) by 30 min pulses at 2 h intervals.

specific for nucleosides (table 3). With respect to the untreated controls, the uptake of ribo- and deoxyribonucleosides, either purinic or pyrimidinic, was immediately inhibited by 70–90%. Conversely, the uptake of the nucleobases tested, and of leucine, choline and glucose, was not affected by the compound. In time course experiments, the uptake of leucine, glucose and choline was always at the control rate (data not shown).

The inhibitory effect of B-31 on nucleoside transport could be antagonized and reversed by increasing concentrations of the nucleoside itself (fig. 5). 100 μM of unlabeled deoxyadenosine and 50 μM of unlabeled deoxyguanosine totally reversed the inhibition by 172 μM(50 μg/μl) B-31 of the uptake of their labeled counterparts. By contrast, up to 1000 μM of unlabeled uridine was necessary to obtain control levels of 3H-uridine incorporation.

Effect on uridine uptake and mitosis of different cell types. The double inhibitory effect of B-31 on nucleoside uptake and mitosis was compared in cell cultures of normal and tumor origin derived from different animal species (table 4). B-31 inhibited the uptake of

Table I. Effect of different treatments with B-31 on the incorporation of labeled uridine into RNA.

B-31 present during		pmoles incorporated after	
Pulse (16 °C)	Incubation (37 °C)	0 min.	30 min.
None	None	-	8.4
None	20 µg/ml	-	9.5
None	-	0.3	-
20 µg/ml	None	-	0.7
20 µg/ml	20 µg/ml	-	0.7
20 µg/ml	-	0.02	-

Exponentially growing HEp2 cells (10^5/ml) were pulse-labeled with ^{3}H-uridine (100 pmoles total) with or without B-31 for 30 min at 16 °C. Monolayers were extensively washed with unlabeled medium and chased at 37 °C with or without B-31.

Table 2. Effect of B-31 on the inhibitory action of BVDU against HSV-1.

Compound	HSV-1
	PFU/ml
None	1×10^7
B-31 25 µg/ml	9×10^6
BVDU 0.3 µM	8×10^3
B-31 25 µg/ml + BVdU 0.3 µM	8×10^6

Confluent monolayers of Vero cells (10^6/plate) were infected with HSV-1 (m.o.i. = 1) for 1 h at room temperature, with or without BVDU alone, B-31 alone or BVDU and B-31 together, at the indicated concentrations. After extensive washings, the cultures were incubated at 35 °C in media containing the same concentrations of the compounds for about 18 h. After freezing and thawing, the virus yield was determined by plaque titrations.

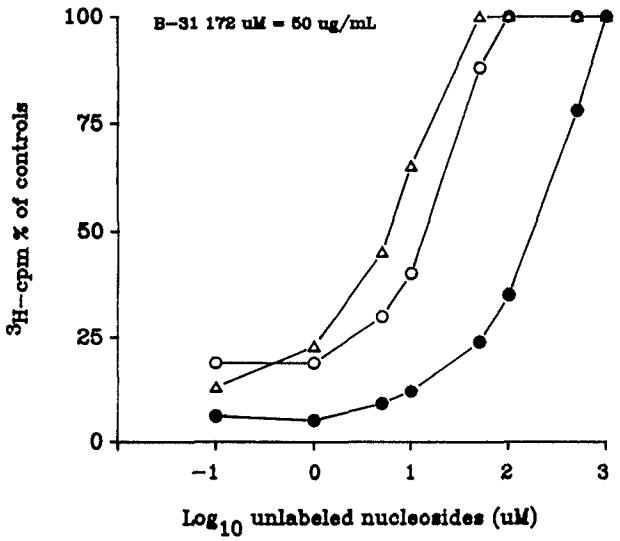

Figure 5. Reversal of inhibition of nucleoside transport by uridine, deoxyadenosine and deoxyguanosine. Duplicate cultures of exponentially growing HEp2 cells (10^5/samples) were incubated in medium containing increasing concentrations of the unlabeled nucleosides and 2 µCi/ml of each corresponding tritium-labeled nucleoside, with or without B-31, 50 µg/ml. After a 10 hour pulse the radioactivity incorporated into RNA was determined. Uridine (—●—); Deoxyadenosine (—○—); Deoxyguanosine (—△—).

Table 3. Effect of B-31 on the uptake of labeled nucleosides, nucleobases, leucine, glucose and choline.

3H-compound	CPM% of controls
TdR	10
CdR	22
AdR	24
GdR	29
UR	14
CR	9
AR	15
GR	15
Adenine	90
Guanine	85
Leucine	100
Glucose	100
Choline	100

Exponentially growing HEp2 cells (10^5/sample) were incubated for 1 h with or without B-31 (50 µg/ml). Pulses with labeled nucleosides and nucleobases were for 1 h with 2 µCi/ml of each. Labeling medium for 3H-glucose and ^{3}H-choline was PBS without serum and pulses were for 2 h with 20 µCi/ml and 10 µCi/ml, respectively.

Table 4. Effect of B-31 on uridine uptake and mitosis in various cell types.

Cell culture	%M-phase cells		CPM% of controls	
	B-31	colcemide	B-31	colcemide
HEp2 (human)	70	80	10	90
HeLa (human)	73	75	7	95
PBMC (human)	75	75	8	90
L929 (mouse)	35	50	20	90
Vero (monkey)	10	15	14	85
CEF (chicken)	17	12	16	85

Exponentially growing cells (10^5/sample) were incubated with or without B-31 (25 µg/ml) or colcemide (0.02 µg/ml). After 1 h a set of cultures was pulse-labeled with ^{3}H-UR as described in the legend to Figure 3. After 24 h another set of cultures was processed for metaphases determination (see 'Materials and methods'). The proportion of M-phase cells in untreated culture was between 5 and 10%.

uridine in all cell cultures by 80–90% whereas colcemide, used as a reference compound, always showed incorporation values similar to those of untreated controls. With respect to its inhibitory effect on cell mitosis, B-31 behaved like colcemide and its action was species-specific. Cells of human origin (HEp2, HeLa and PBMC) were the most sensitive, showing 70–80% of metaphase-arrested cells after 24 h of treatment with either B-31 or colcemide. The murine L929 cells were less sensitive (35% and 50% of M-phase cells with B-31 and colcemide, respectively), whereas the monkey cells (Vero) and the chicken fibroblasts (CEF) appeared almost completely resistant to the antimitotic action of both compounds.

Discussion

B-31, previously described as an antimitotic compound[10], is here shown to be a potent inhibitor of the

transport of nucleosides across the cell membrane. The inhibitory effect at the membrane level is specific since the uptake of leucine, choline, glucose, and nucleobases was unaltered even in the presence of the maximum non-toxic dose of B-31 (25 μg/ml).

The upake of any nucleoside is strongly reduced but the inhibitory effect of B-31 appears stronger against the pyrimidinic molecules than against their purinic counterparts: 1 μg/ml (about 3 μM) reduced the uptake of uridine by 50%, whereas a 10-fold higher dose was necessary to achieve the same degree of inhibition of purinic nucleoside uptake (data now shown).

The inhibition is fully reversible either by removal of B-31 from the cultures, or by addition of high concentrations of the nucleosides themselves. With deoxyguanosine a complete reversal was obtained with one third of the B-31 dose, whereas a concentration 5-fold higher than that of B-31 was necessary to reverse the inhibition of uridine uptake, indicating in this case a higher affinity of the compound for the transporter(s).

The nucleoside transport system inhibited by this pyrimidine analogue remains to be established. However, since the nucleoside uptakes of human, monkey, mouse or chicken-derived cells were inhibited to the same extent, the target of B-31 must be present in most if not all animal cells. To date, 2 types of facilitated-diffusion systems and 2 Na+-dependent nucleoside transporters have been characterized[4]. Facilitated-diffusion systems sensitive to NBMPR (a potent nucleoside transport inhibitor) have been described in most mammalian cells[7], including HeLa[1,6], whereas the Na+ coupled (concentrative) systems have been characterized in a variety of animal but not in human cells[1,9].

In its mitostatic effect, B-31 behaves very much like colcemide, showing a species-specificity of action. Human cells, whether epithelioid, fibroblasts or lymphocytes, appear the most sensitive. Murine cells are less sensitive, and monkey and chickens cultures are resistant to the antimitotic action of both compounds. These results agree with those obtained by Gupta[2] with a number of anti-microtubular compounds (colchicine and vinblastine among them) in a study of their species-specific differences of action. This author ascribed the observed differences of cell sensitivity to a minor or major ability of the compounds to enter the cell itself. If this also holds true for B-31 (the similiarities between B-31 and colchicine molecules are evident from their structural formulae), it may well explain why its mitostatic effect is species-specific whereas the inhibition of nucleoside transport is not.

Nevertheless, the possibility that B-31 has only one target in the cell and that the 2 inhibitory effects are related to each other cannot be excluded. The cytoskeleton has been identified as the mediator of interactions on the cell surface between the transport systems for anions, cations and sugars[3], and colchicine has been reported to interfere with the uptake of prolactin and insulin in rat liver cells[8]. Studies are in progress to establish whether B-31 is able to discriminate between the high and low NBMPR-sensitive nucleoside transporters and to determine the Na+-dependence of its inhibition of nucleoside uptake.

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** Abbreviations. BVDU, 5-bromovinyl-2′-deoxyuridine; HSV-1, herpes simplex virus type 1; HIV-1, human immunodeficiency virus type 1; NBMPR, nitrobenzythioinosine, 6-[(4-nitrobenzyl)thio]-9-8-D-ribofurannosylpurine.

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