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Relevance of DNA binding to the mechanism of anti-herpesvirus activity of benzhydrazone

Giorgio Palu'[a], Mauro Tognon[c], Maria Grazia Romanelli[d], Mario Rassu[a], Maria Cristina Parolin[a], Giuseppe Zagotto[b] and Manlio Palumbo[b]

[a]*Institute of Microbiology and* [b]*Department of Pharmaceutical Sciences, University of Padova, Padova, Italy,* [c]*Institute of Histology and General Embryology, University of Ferrara, Ferrara, Italy and* [d]*Institute of Biological Sciences, University of Verona, Verona, Italy*

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Summary

Benzhydrazone (1H-benz(f)indene-1,3(2H)-dione bis (amidino-hydrazone) (BH) is a synthetic compound with selective anti-herpesvirus activity. Its selectivity seems to stem from the inhibition of viral protein glycosylation and several hypotheses have been formulated to explain such an effect. Data presented here demonstrate that DNA binding is a prominent feature of BH. Interaction is taking place with a relatively high affinity constant and is more efficient for GC-rich viral sequences. Experiments with the cloned DNA fragments from a BH-resistant virus strain indicate that BH-DNA complex formation is drastically reduced as compared to BH-sensitive virus. The occurrence of the resistant phenotype in HEp-2 cells but not in Vero cells could be explained by differences in BH cytotoxicity. Changes in drug uptake and accumulation by cells following infection, in addition to GC preference, may also account for the degree of antiviral selectivity shown by BH.

DNA-binding; Drug-resistance; HSV; Benzhydrazone

Introduction

1H-benz(f)indene-1,3(2H)-dione bis (amidino-hydrazone), named benzhydrazone (BH) (Fig. 1), has been described as an inhibitor of the glycoprotein

Correspondence to: G. Palu', Institute of Microbiology, University of Padova, Via A. Gabelli 63, 35121 Padova, Italy. Fax: 49-8750012.

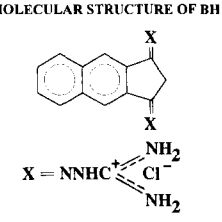

Fig. 1. Structural formula of benzhydrazone.

biosynthesis of herpes simplex viruses (HSV). The effect of this compound seems to be specific for HSV in as much as BH does neither affect the glycosylation of other viruses nor that of uninfected cells.

Recently, we have demonstrated that BH-sensitivity (BH^S) is genetically determined in HSV and that the compound selectivity may depend on an as yet unidentified viral function. Pertinent to these observations were the isolation of a BH-resistant (BH^R) HSV mutant, named HSV-1(13)S11 and the genetic transfer of the resistant trait to BH^S HSV strains (For a review see Roizman & Batterson). This result was obtained by marker transfer experiments using two DNA fragments, *Bam*HI L and *Bam*HI SP (Tognon et al., 1988).

In a recent theoretical investigation, mostly focussed on a stereochemical comparison between BH and tunicamycin, we have ruled out the possibility that BH might directly inhibit a virus-specific glycosylation step (Paganetto et al., 1989).

In an attempt to further elucidate the mechanism(s) whereby BH inhibits HSV replication and, hopefully, to understand the relationship between interference with glycosylation and anti-HSV selectivity, we have evaluated the hypothesis (Tognon et al., 1984; Roizman & Batterson, 1985; Tognon et al., 1988) that BH does not penetrate in uninfected cells but only in HSV-infected cells after the plasma membrane has become leaky. Additionally, from a general appraisal of the BH molecule, which is made up of an aromatic moiety and a positively charged side chain group, we have considered the possibility of an interaction with nucleic acids, in particular DNA.

Specifically, in this paper we present quantitative data on BH cytotoxicity, antiviral activity and penetration into different cell lines, either mock-infected or infected with the sensitive and the resistant virus strains. In addition, we report a study on the comparative interaction of BH with several cellular and viral DNAs, including the HSV DNA fragments which represent the loci of the genome where the BH^R mutations were mapped.

Materials and Methods

Compounds

1H-benz(f)indene-1,3(2H)-dione bis (amidino-hydrazone), named benzhydrazone (BH), was synthesized and purified following a previously described

method (Cavrini et al., 1979). Stock solutions (10 mM) of the drug were made in sterile distilled water. An extinction coefficient of 27 000 $M^{-1} \times cm^{-1}$ at 280 nm was used. Solutions were aliquoted and kept at −20°C until used. [^{35}S]Methionine (>400 Ci/mmol) was purchased from AmityPG (Milan, Italy). All reagents were AnalaR grade.

Cells and viruses

HEp-2 and Vero cells were routinely grown in Dulbecco's modified minimal essential medium (DMEM) supplemented with 2 mM fresh glutamine, 20 mM HEPES, antibiotics and 5% (v/v) foetal calf serum (FCS). The BH-sensitive and -resistant strains of HSV-1, named HSV-1(13) and HSV-1(13)S11, respectively, have already been described (Manservigi et al., 1974; Tognon et al., 1984; Tognon et al., 1988). Briefly, mutant HSV-1(13)S11 was obtained from the parental strain HSV-1(13) after continuous selection with 100 μg/ml of 5-bromo-2'-deoxyuridine (BUdR). The mutant strain was selected upon its syncytial plaque morphology that differed from the cytoaggregating plaque morphology (cyt) of the wild type virus. Glycoprotein synthesis of the mutant strain was not inhibited by BH.

Antiviral activity

Drug sensitivity testing was performed in vitro by a conventional plaque assay measuring reduction of the infectious titer and of the virus yield. Experiments were carried out essentially as already described (Palù et al., 1984). Briefly, confluent monolayers of Vero cells and HEp-2 cells, inoculated with the two virus strains at a m.o.i. of 10^{-4}–10^{-2} PFU/cell, were exposed to different drug concentrations. Plaques were either enumerated directly (after 48 h) or in new drug-free monolayers inoculated with the lysates of the original cultures. The concentration of the drug required to reduce plaque formation by 50% (EC_{50}) or 90% (EC_{90}) was deduced as previously reported (Palù et al., 1984).

Cytotoxicity studies

The cell growth inhibitory effects of BH were determined in exponentially growing Vero and HEp-2 cells over a period enabling at least three cell divisions. Cells were counted after trypsinization of the monolayers by a conventional hemocytometer. Values for CC_{50} and CC_{90} were obtained as already described (Palù et al., 1984).

Permeation studies

HEp-2 and Vero cells were mock-infected and infected with the wild type and the mutant viruses at a multiplicity of 20 PFU/cell. After the adsorption period, cell monolayers were incubated at 37°C for 16 h in the presence of 30 μM BH in DMEM containing 2% FCS. At the end of the incubation period, cells were extensively washed with isotonic water and the drug extracted with 0.1 M HCl in 50% ethanol, as previously reported (Bachur et al., 1970). In

experiments dealing with BH uptake and distribution in subcellular compartments, uninfected HEp-2 and Vero cells were incubated with 30 μM BH for a period of 2 h. Cells were extensively washed as before, scraped from the plastic surface with a rubber policeman and incubated in hypotonic buffer (Palù et al., 1986) at 4°C for 10 min under mild agitation. Cells were broken by passage through a 19 and a 23 gauge needle and the nuclei, cytosol and membrane components were obtained essentially as described earlier (Palù et al., 1986). Drug was extracted as above.

Inhibition of protein synthesis

HEp-2 cells were mock-infected or infected with the HSV-1(13) or HSV-1(13)S11 strains at a multiplicity of 20 PFU/cell and labelled with methionine for 2 h intervals starting from 1 h post-infection (p.i.) up to 5 h p.i. Protein profiles were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5 to 15% gradient gels as already described (Tognon et al., 1984; Tognon et al., 1988). Once fixed and dried gels were exposed for autoradiography and scanned with an AMBIS beta scanner equipment. This procedure allows to quantitate the radioactivity present in each single protein band and, hence, provides information on the relative inhibition of host cell protein synthesis which occurs temporarily after infection.

Preparation of viral and cellular DNA

Viral DNA was obtained from confluent monolayers of Vero cells infected with HSV-1(13) or HSV-1(13)S11 strains at a multiplicity of 0.01 PFU/cell, once cytopathic effect encompassed 100% of the whole cell population. Extraction and purification of the nucleic acid were performed according to the method of Walboomers and Ter Schagget (1976). The *Bam*HI L and *Bam*HI SP fragments (BH resistance regions) together with the *Bam*HI Q fragment (control sequence) were originally obtained from the genome of the two viruses after digestion with *Bam*HI and purification by means of conventional agarose gel electrophoresis and electroelution techniques (Tognon et al., 1988). The fragments were then cloned in the *Bam*HI site of pUC plasmids that were propagated in JM 83 *E. coli* cells. The DNA corresponding to the viral fragments was finally obtained by massive plasmid preparations (Tognon et al., 1988), *Bam*HI digestion, electrophoresis and electroelution as above. Calf thymus DNA (from Sigma) was sonicated up to a Mr of 400 000 and used as a further control.

Binding studies

Spectrofluorometric measurements took advantage of the high emission efficiency of BH when irradiated at 330 nm and of the dramatic effects caused by DNA addition. The changes in fluorescence response could be easily related to the concentration of free and DNA-bound drug. A Perkin-Elmer MPF66 apparatus, interfaced to a PE 7500 data station was used. One to 40 scans were

accumulated according to sample response and concentration. Unless stated otherwise, the physicochemical measurements were carried out at 25°C in aqueous 10 mM Tris-HCL buffer (pH 7.0), containing 1 mM EDTA and known amounts of sodium chloride to adjust ionic strength to the desired value (ETN buffer). Experiments were performed by addition of known amounts of DNA to solutions containing a given concentration of the ligand, until saturation was reached. Total concentration of both the ligand and the macromolecule were determined by adsorption measurements. Experimental binding data were plotted according to the method of Scatchard (1949) and analyzed using the neighbor exclusion model, which describes the binding of a ligand to a homogeneous lattice, according to the equation:

$$r/m = K_i (1 - nr) (1 - nr)/[1 - (n - 1)r]^{n-1}$$ (McGhee & Von Hippel, 1974)

where r is the number of ligand molecules bound per DNA phosphate, m is the free ligand concentration, K_i is the intrinsic binding constant, and n is the number of consecutive lattice residues covered by a ligand molecule. A best fitting least squares procedure (Marquardt fit) was used.

Results

Cytotoxicity and antiviral activity

The antiviral activity and the cytotoxic potential of BH are presented in Table 1 as the respective EC_{50}, EC_{90}, CC_{50} and CC_{90} values. As it can be appreciated from inspection of Table 1, the HSV-1(13)S11 mutant strain was less sensitive to BH than the parent strain HSV-1(13) only by an order of 2–6 fold. Inhibition of viral growth did not vary significantly (less than 20%) when

TABLE 1

BH inhibition of virus and cell growth

Virus Growth	Ec_{50} (μM)				EC_{90} (μM)			
	Infectious titer reduction		Virus yield reduction		Infectious titer reduction		Virus yield reduction	
	Vero	HEp-2	Vero	HEp-2	Vero	HEp-2	Vero	HEp-2
HSV-1(13)	2±0.3	2.5±0.5	1.5±0.2	2±0.4	15±2	18±3	8.5±1.5	8±2
HSV-1(13)S11	13±2	16±4.5	5±0.8	6±1.5	35[a]±7	32±8	15±3	16±4

Cell Growth	CC_{50} (μM)	CC_{90} (μM)
Vero	18±2.5	130±15
HEp-2	35±8	175±20

[a]Cytotoxicity was noted at this concentration.

Values reported represent the average of three sets of different experiments performed in triplicate.

TABLE 2

BH uptake by Vero and HEp-2 cells in the course of the infection with BH^S wt HSV-1 (13) and BH^R mutant HSV-1 (13) S11 strains[a]

	BH nmol/10^6 cells		
	Control	HSV-1 (13)	HSV-1 (13) S11
HEp-2	8.5 ± 1.6	11.5 ± 2	10.1 ± 1.1
Vero	5.5 ± 1.1	7.8 ± 1.4	7.2 ± 1.3

[a]Cells were infected at a multiplicity of 20 PFU/cell and incubated in the presence of 30 μM BH for 16 h at 37°C. Values reported represent the average of three sets of different experiments performed in triplicate.

Vero cells or HEp-2 cells were used as the host. In the case of strain HSV-1(13), the BH selectivity index did not exceed 10 (or 20, according to the host cell employed). BH, in fact, exhibited a more prominent growth inhibitory effect on Vero cells as compared to HEp-2 cells (about 2-fold).

Drug incorporation and effect of virus infection

Following infection by HSV-1(13) BH^s and HSV-1(13)S11 BH^R strains, cells became significantly more permeable to BH (25–45% difference with respect to controls) (Table 2). The compound was accumulated to a greater extent by HEp-2 cells than by Vero cells (50% difference). In these experiments, characterized by a long period of drug exposure (16 h), BH accumulation was prevalent in the nuclear compartment (> 60%) of both cell lines (not shown). BH uptake was slightly (but not significantly) higher in both cell populations following infection with the wt BH^s strain as compared to the mutant BH^R one. Absolute number of intracellular molecules was about 10^{10} molecules per cell.

Inhibition of host cell protein synthesis by HSV-1(13) BH^S and HSV-1(13)S11 BH^R strains

Inhibition of host cell protein synthesis was followed in HEp-2 cells infected at a multiplicity of 20 PFU/cell up to 7 h p.i. Both viruses caused a similar inhibition of protein synthesis in HEp-2 cells.

A complete shutoff, however, was not reached even at 7 h p.i., in agreement with published data on the behavior of HSV-1 (Kwong & Frankel, 1989). Notwithstanding a virtually identical decline in the production and accumulation of host polypeptides which was induced by the viral infection, the two strains exhibited a quite distinct protein profile, which is in keeping with previous results (Tognon et al., 1984). Individual differences between the wt and the BH^R mutant, particularly evident at the level of gC and its precursor (pgC) and of other proteins (mainly ICPs 23, 32, 35, 43 and 44), have already been reported. They have been attributed to intra-strain variability for ICP44 and to randomly occurring unidentified point mutations caused by BUdR mutagenesis. In fact, comparative analysis with several restriction endonu-

cleases failed to show any difference between DNAs from the two strains (Tognon et al., 1984; Romanelli et al., 1991).

DNA binding ability of BH

BH exhibited an absorption maximum at about 300 nm in aqueous buffer solution. The compound was also endowed with a relatively high intrinsic fluorescence with a broad emission centered at 420 nm. Substantial changes of the spectroscopic response of BH occurred upon addition of DNA to buffered solutions of the drug. The absorption spectrum underwent both slightly hypochromic and bathochromic modifications in the presence of the nucleic acid (Fig. 2A). Further evidence for a binding process was obtained by

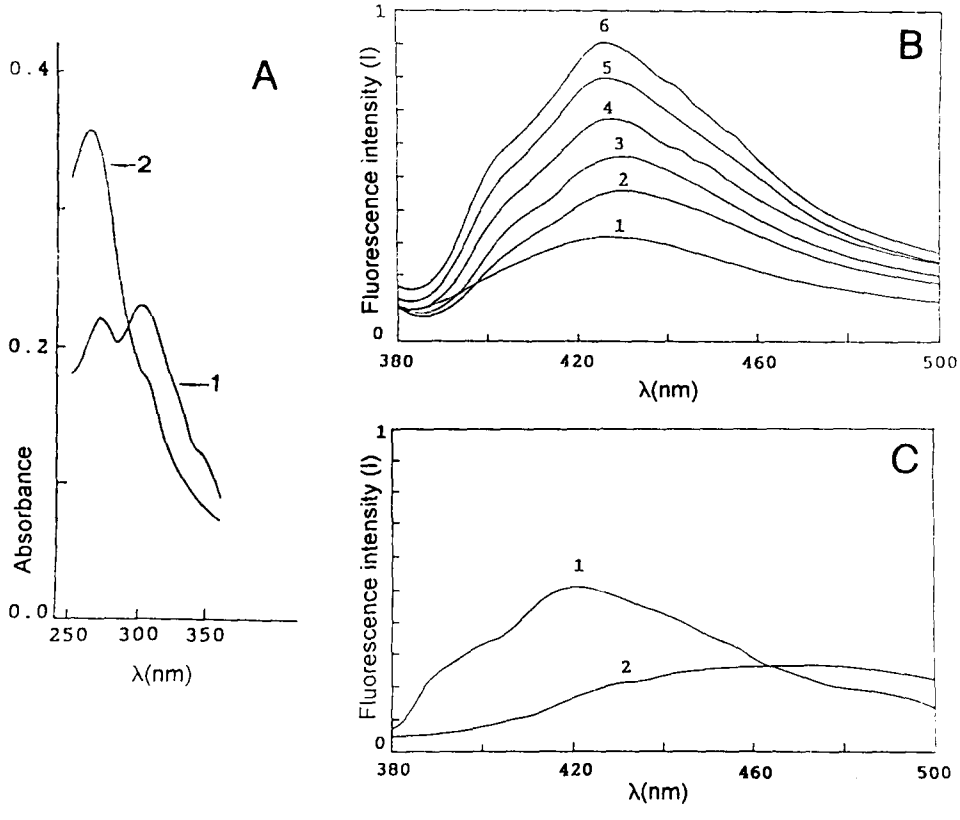

Fig. 2. A. Absorption spectrum of BH in the ultraviolet region in the presence/absence of calf thymus DNA. (1) BH 8 μM in ETN 22 mM pH 7; (2) BH 8 μM in the presence of 40 μM nucleic acid (on a phosphate molar basis). Fig. 2B. Fluorescence emission spectra (excitation at 330 nm) of BH 2.4 μM in ETN 22 mM (pH 7), in the presence of various amounts of *Bam*HI L S11. P/D represents the DNA/drug molar ratio. (1) No DNA; (2) P/D = 1.2; (3) P/D = 2.4; (4) P/D = 4.7; (5) P/D = 7; (6) P/D = 11.8-25. Fig. 2C. Fluorescence emission spectra (excitation at 330 nm) of BH 2.4 μM in ETN 22 mM (pH 7), in the presence of various amounts of *Bam*HI SP 13. P/D represents the DNA/drug molar ratio. (1) No DNA; (2) P/D 1.2-11.3.

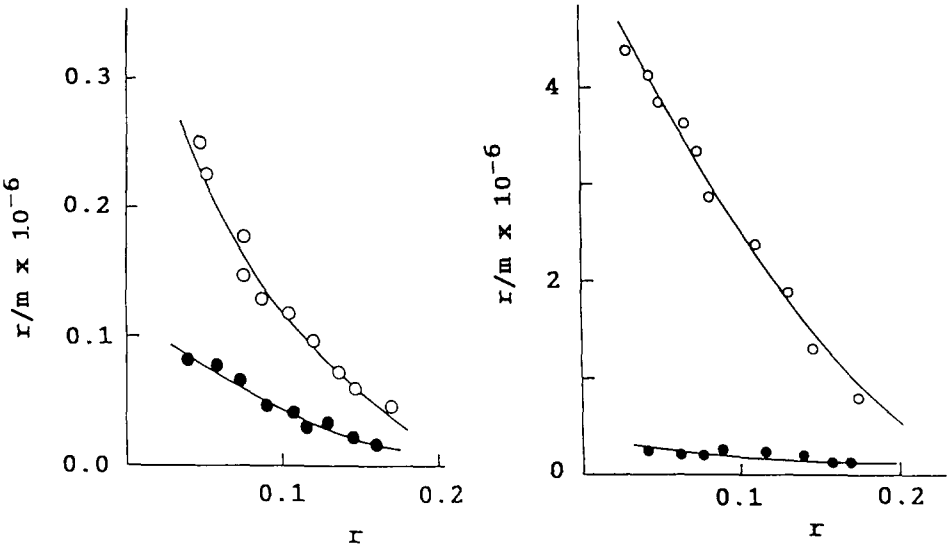

Fig. 3. Left panel. Scatchard plot for the interaction of BH with *Bam*HI L 13 (open circles) and *Bam*HI L S11 (full circles) in ETN 22 mM (pH 7), and 25°C. Right panel. Scatchard plot for the interaction of BH with *Bam*HI SP 13 (open circles) and *Bam*HI SP 11 (full circles) in ETN 22 mM (pH 7), and 25°C. *r* represents the bound drug/total DNA ratio; *m* represents the free drug concentration.

fluorescence measurements. In fact, the emission response of BH was dramatically affected upon addition of the nucleic acid, as shown in Fig. 2B. The results were strongly influenced by the nucleic acid sequence being examined, as an indication that the drug must have different sites of binding. An increase in the drug quantum yield was observed for the *Bam*HI L fragments as opposed to a decrease in the case of the *Bam*HI SP and *Bam*HI Q fragments. In particular, the complex between BH and *Bam*HI SP showed a considerable red-shift of the emission maximum (Fig. 2C). The fluorometric studies enabled to draw the Scatchard plots for the binding process (Fig. 3), from which the intrinsic constants were evaluated. The results for all tested fragments are summarized in Table 3. BH possessed a reduced affinity for the drug resistant regions of the mutant virus. The decrease was nearly 3-fold for the *Bam*HI L fragment and more than one order of magnitude for the *Bam*HI SP fragment. The exclusion coefficient value *n* was constant, indicating that two base pairs were involved in the interaction between DNA and the ligand. No cooperativity phenomena were occurring in the binding process. The data were obtained at relatively low ionic strength. Although it can be expected that the intrinsic binding constant be somewhat lower at physiological salt concentration as BH is a charged species, the relative affinity scale for the various fragments should not exhibit appreciable differences. Interestingly, when the *Bam*HI Q fragments were utilized, as a control for differences in binding affinity, no real variation distinguished the mutant from the wild type sequences. However, the absolute affinity exhibited by BH for the *Bam*HI Q

TABLE 3

Binding parameters of BH to various DNA fragments in ETN buffer, 25°C and pH 7.0

Fragment	$K_i \times 10^{-6}$	n (bp)
*Bam*L 13	0.35 ± 0.08	2.1 ± 0.1
*Bam*L S11	0.12 ± 0.03	1.9 ± 0.1
*Bam*SP 13	5.4 ± 0.1	2.0 ± 0.1
*Bam*SP S11	0.43 ± 0.08	2.1 ± 0.2
*Bam*Q 13	0.05 ± 0.02	n.d.
*Bam*Q S11	0.05 ± 0.02	n.d.

n.d. = not determined.
See text for definition of K_i and n.
Values reported represent the average of measurements performed at least in triplicate.

fragment was considerably lower than the affinity for fragments where the BH^R mutation has been assigned.

Discussion

A number of mechanisms have been proposed to explain the action of BH, a selective inhibitor of the synthesis of herpes simplex virus glycoproteins (for a review see Roizman & Batterson, 1985).

In a previous investigation, we were able to exclude the possibility that BH acts as a metabolic inhibitor of glycoprotein biosynthesis (Paganetto et al., 1989). However, looking back to literature data (Campadelli-Fiume et al., 1980; Serafini-Cessi & Campadelli-Fiume, 1981), we appreciated that a proper assessment of the antiviral selectivity of BH had never been done. As we have shown in the present paper, the therapeutic index of BH is only moderate (10–25), being greater in HEp-2 cells for they are more resistant than Vero cells to the drug's is cytotoxic action. Moreover, the 30 μM dose used for selection of resistance is higher than the CC_{50} value for Vero cells (Table 1B). This explains why the BH^R phenotype could not be observed in this cell line (Tognon et al., 1984). The cytotoxic potential of BH would thus point to a target of action which is common to both the host cell and the virus.

From data on cellular drug incorporation it is evident that mock-infected and virus-infected HEp-2 cells are accumulating relatively more drug than Vero cells under similar conditions, with a prevalent nuclear distribution. One should then reject the proposal that the BH^R phenotype arises exclusively in HEp-2 cells as the result of lack of cell permeation (Tognon et al., 1984). Furthermore, BH resistance can not possibly originate from lack of permeation promoted by the HSV-1(13)S11 mutant, as virtually comparable levels of intracellular BH are present in cells infected with the two strains. HSV-1(13) wt and its mutant derivative also do not differ in their ability to inhibit host cell protein synthesis.

Thus, virus sensitivity or resistance to BH cannot be explained by differential effect on host protein shutoff (Carrasco, 1978; Hohn, 1985; Castrillo &

Carrasco, 1986; Palù et al., 1990) brought about by the two strains.

As it could be suggested on the basis of its structural analogy with some DNA binding agents (Paganetto et al., 1989; Palù et al., 1987), BH is able to bind efficiently to the nucleic acid. Although the mode of interaction has not been investigated in detail, a few points deserve to be made. The structural and electronic properties of BH are compatible both with intercalative and external binding to DNA (Wilson et al., 1989; Larsen et al., 1991). Since non-intercalators are A-T specific and exhibit exclusion coefficients higher than 2 bp (Pullman, 1989), our data suggest at least a partial intercalation of BH between DNA base pairs. Interestingly, the fluorescence response is modified in a opposite manner when using the *Bam*HI L or SP fragment (Fig. 2B and 2C). This fact points to a different geometrical arrangement of the drug when bound to DNA of either sequence. The affinity constant for complex formation between BH and the *Bam*HI fragments is in the range of 10^6–10^5 M^{-1} in all cases, a value well comparable to those reported for drugs known to act on DNA such as anthracycline antibiotics (Arcamone, 1981). It is clearly related to the DNA sequence being examined, in particular its G-C content. BH in fact, appears to be characterized by a preferential binding to the *Bam*HI SP fragment (78% G-C) in comparison to the L and Q sequences, which exhibit a lower G-C content (71% and 63%) (McGeoch et al., 1985; McGeoch et al., 1988). This fact is further confirmed by the remarkable decrease in affinity of calf thymus DNA (40% G-C) (data not shown). Differences in binding affinity between HSV-1(13) and HSV-1(13) (S11) DNAs were only observed for *Bam*HI L and *Bam*HI SP fragments and not with genomic sequences which are not coding for BH resistance. Since the fragments *Bam*HI L and *Bam*HI SP represent the loci involved in BH resistance, mutational events that affect DNA binding, although not revealed by restriction mapping (Romanelli et al., 1991), are most likely related to drug resistance. Thus, complex formation with DNA is in agreement with the differences in antiviral activity (Table 1) and must be a distinctive feature of the mode of action of BH. The notion that DNA is a target of BH could help in understanding why products of late genes, such as structural glycoproteins, that are mainly expressed after viral DNA synthesis has been taking place, can be selectively inhibited (Conley et al., 1981). BH may represent a tool to unravel key steps in the regulation of the HSV replicative cycle.

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