Antimicrobial Activity and Mechanism of Action of Indolo-(2,3-b)Quinoxaline and Analogs

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The antiparvovirus activity of 14 derivatives of indoloquinoxaline was tested. The most active was 2,3-dimethyl(dimethylaminomethyl)5H-indolo-(2,3-b)quinoxaline, also called B-220. The antiparvovirus mechanism of B-220 was sought. The compound inhibited replication of herpes simplex virus type 1, cytomegalovirus, and varicella-zoster virus in tissue culture at concentrations of 1 to 5 μM, depending on the cell type used for assay and the amount of virus. Cellular toxicity was seen at a concentration of 10 to 30 μM, and antiparvovirus activity in the human bladder cancer and human embryonic lung fibroblast cell lines tested was found at concentrations of 3 to 15 times lower than the concentrations causing cellular toxicity. Viral DNA synthesis, as well as production of early and late viral proteins, was inhibited at 0.5 to 4.5 μM B-220, but viral DNA polymerases tested in vitro were not inhibited at these concentrations. There was no interaction with the pyrophosphate analog ofospacarn, and no reversal of the antiparvovirus activity of B-220 occurred with naturally occurring nucleosides. We conclude that the antiviral effect depends on the multiplicity of infection and may occur at the level of viral DNA synthesis and that no interference occurs with pyrophosphate analogs or nucleosides. The more potent activity against viral DNA than against cellular DNA may be caused by a true selectivity for herpesvirus DNA or by the higher metabolism of viral DNA in infected cells.

Several condensed-ring systems containing indole have antiparvovirus activity (6). Such compounds are distantly related to ellipticin and quinacrine, which are known to intercalate in DNA (for a review, see reference 11). Compounds in the 6H-indolo-(2,3-b)quinoxaline series were synthesized, and their effect on herpesviruses was assayed in different cells. The most active compound, 2,3-dimethyl(dimethylamino)methyl)5H-indolo-(2,3-b)quinoxaline (B-220), was further tested for antiviral effect and mechanism of action. Indoloquinoxalines in high concentrations (around 300 μM) inactivate virions, while at lower concentrations (around 3 μM), synthesis of viral DNA and protein appears to be reduced. B-220 was tested against herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV), and varicella-zoster virus (VZV). A comparison was made with three previously known active substances: acyclovir (ACV; 3) for HSV-1, phosphonoformate (PFA; 15) for CMV, and the guanosine analog (RS)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (1) for VZV.

MATERIALS AND METHODS

Compounds. B-220 (Fig. 1) and related derivatives of the B series (Table 1) were synthesized at the Department of Chemistry, Royal Institute of Technology, Stockholm, Sweden. PFA, phosphonoacetic acid (PAA), and (RS)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (1) were kind gifts from Bo Öberg, Astra AB, Södertälje, Sweden. The PAA and PFA salts of B-220 were made at the Royal Institute of Technology. Nucleosides and nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.). Trinitated thymidine and tritiated thymidine triphosphate were from Dupont, NEN Research Products (Boston, Mass.). Activated calf thymus DNA or synthetic polymers (Collaborative Research, Inc., Waltham, Mass.) were used as templates.

Virus. HSV-1 strain C42, CMV strain Ad.169, and VZV strain ULF were grown in human embryonic lung (HL) cells. CMV strains V4078 and 2V2828 were only partially susceptible to PFA and were used after passages in PFA-containing medium.

Cells. HL cells, rabbit cornea cells (Sirc cell line), human embryonic rhabdomyosarcoma cells, A549 (a human lung cancer cell line), Vero and AGMK cells (from African green monkey kidney), rabbit kidney cells (RK-13), baby hamster kidney cells (BHK-21), HeLa cells (from human cervical cancer), and EJ cells (from human bladder cancer) were propagated in our laboratory. All cells were maintained in Dulbecco modified Eagle medium with 5% fetal calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml). All cell cultures were free of mycoplasma contamination.

Cellular toxicity. The toxicity of each compound was determined on the basis of [3H]thymidine (Amersham Corp., Arlington Heights, Ill.) incorporation into cellular DNA (8) or by trypan blue staining. The assays were performed on established contact-inhibited monolayers.

Virus susceptibility assay. Susceptibility of the viruses to antiviral compounds was determined as described elsewhere (14). After 48 to 72 h of culture of each virus in the presence of different concentrations of drug, viral protein production was quantified by a virus protein-specific enzyme-linked immunosorbent assay (ELISA; 13). The 50% infective concentration (IC50) was defined as the drug concentration needed to reduce viral protein production by 50%.

HSV-1, VZV, and CMV DNA polymerases. Roller bottles with confluent monolayers of HL cells were infected with HSV-1, CMV, or VZV. When the cells showed 50 to 70% cytopathic effect, they were washed with phosphate-buffered saline and collected. DNA polymerase was isolated by separation on DEAE and phosphocellulose columns according to published methods (10, 12). Viral DNA polymerase activity was assayed in 100-μl reaction mixtures containing 100 mM Tris hydrochloride (pH 8.0), 20 mM MgCl2, 2.5 mM

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dithiothreitol, 45 μg of bovine serum albumin, 100 mM KCl, 0.1 mM each dATP, dCTP, and dGTP, and 1 mM dTTP. About 0.005 mM [3H]dTTP (1,000 to 5,000 cpm/pmol) was used. Activated calf thymus DNA (20 μg) was used as the template to determine cellular HSV-1 and CMV polymerase activities; oligo(dA)/poly(dT) was used to measure VZV polymerase activity. Viral polymerase solution (20 μl) was added to the assay, and the mixture was incubated for 30 min. Incorporated [3H]dTTP was measured in a scintillation counter.

**CMV DNA hybridization.** Hybridization of CMV DNA and application of the procedure to antiviral compounds have been described (5).

**Immunofluorescence.** Staining for CMV early antigens was performed by anticomplement immunofluorescence (ACIF; 15) 1 day after infection. The serum was a rabbit anti-CMV DNA polymerase (ACIF titer, 1:80). Late CMV antigens were determined on day 6 by indirect immunofluorescence, using a monkey anti-CMV nucleocapsid immunoglobulin G (immunofluorescence titer, 1:160).

**RESULTS**

**Structure-activity relationship.** B-220 showed the highest antiviral activity of the compounds tested. The length of the alkyl chain was important (Tables 1 and 2). Extension or reduction by one methylene group reduced the antiviral activity. If the original methyl groups in the R1 and R2 positions were replaced with hydrogens, the antiviral activity also decreased. Many attempts were made to replace the dimethylamino end group (R3) of the alkyl chain.

**TABLE 1. Chemical structures of the compounds tested**

| Compound | X          | R1          | R2          | R3          | R4          |
|----------|------------|-------------|-------------|-------------|-------------|
| B-220    | 2          | CH₃         | CH₃         | N(CH₂)₃     | H           |
| B-220-PFA| 2          | CH₃         | CH₃         | N(CH₂)₃     | H           |
| B-220-PAA| 2          | CH₃         | CH₃         | N(CH₂)₃     | H           |
| B-300    | 1          | CH₃         | CH₃         | 2-Imidazolyl| H           |
| B-301    | 1          | H           | H           | 2-Thiazolyl | H           |
| B-303    | 1          | H           | H           | 2-Imidazolyl| H           |
| B-304    | 2          | H           | H           | N(CH₂)₃     | Br          |
| B-309    | 1          | H           | H           | 2-Benzothiazolyl| Br    |
| B-310    | 1          | CH₃         | CH₃         | 2-Benzimidazolyl| Br    |
| B-311    | 3          | CH₃         | CH₃         | 1-Piperidyl | H           |
| B-317    | 2          | H           | H           | N(CH₂)₃     | H           |
| B-318    | 2          | H           | H           | N(CH₂)₃     | CH₃         |
| B-320    | 1          | H           | H           | N(CH₂)₃     | H           |
| B-321    | 3          | CH₃         | CH₃         | N(CH₂)₃     | H           |

**TABLE 2. Anti-HSV-1 activity of B-220 and analogs in E.J. cells**

| Compound | IC₅₀ (μM) |
|----------|-----------|
| B-220    | 1.51      |
| B-220-PFA| 1.15      |
| B-220-PAA| 1.42      |
| B-300    | 3.24      |
| B-301    | >100      |
| B-303    | 9.66      |
| B-304    | 4.18      |
| B-309    | NS        |
| B-310    | NS        |
| B-311    | NS        |
| B-317    | 4.57      |
| B-318    | 3.88      |
| B-320    | 25.6      |
| B-321    | 4.7       |
| ACV      | 0.3       |

*NS. Not soluble.

Some of the resulting compounds could not be tested for antiviral activity because they were insoluble in water. If the hydrogen in the R4 position was replaced with a methyl group or with bromide, the antiviral activity decreased. The importance of the two nitrogens in the six-member ring is illustrated by the fact that replacement of one of the nitrogens with carbon lowered the antiviral activity (data not shown).

**Toxicity of B-220 in cell culture.** The influence of B-220 on cellular DNA synthesis as indicated by [3H]thymidine incorporation was studied. Nine cell types were tested (Fig. 2). B-220 inhibited cellular DNA synthesis in all of the cell types. E.J., RK-13, and A549 cells were found to be the least susceptible to B-220 inhibition and were selected for further investigation.

**Anti-HSV-1 activity of B-220 and analogs.** The antiviral activities of B-220, B-300, and B-301 were assayed against HSV-1 in three cell types (Table 3). B-220 and B-300 showed higher antiviral activities in E.J. and A549 cells than in RK-13 cells. B-220 showed higher activity than B-300 in all cell types. B-301 exhibited no antiviral activity in any cell type.

The results shown in Fig. 2 and Table 3 indicated that B-220 exhibited the highest antiviral activity when compared with other antiviral activity when HSV-1-infected E.J. cells were used. Fifteen analogs of B-220 were therefore tested in E.J. cells (Table 2). B-220 showed the highest activity against HSV-1. To increase the activity of B-220, the PFA and PAA salts of B-220 were constructed. These salts did not show higher antiviral activity than did B-220. The ratio of B-220 effects on HSV-1 replication compared with cellular toxicity varied between 3 and 15, depending on cell strain and virus multiplicity.

**Antiviral activity of B-220 and B-300 in the presence of nucleosides.** The ability of eight naturally occurring nucleosides to reverse the antiviral activity of B-220 and B-300 was studied in HSV-1-infected E.J. cells. A 100-μM concentration of each of the eight nucleosides was added together with various concentrations of B-220 or B-300 to HSV-1-infected cells at the time of infection. After 48 h of incubation, virus protein production was quantified by ELISA. The results were compared with those obtained for similarly treated cells with no addition of nucleosides. A 100-μM concentration of thymidine is known to reverse the antiviral activity of the nucleoside analog ACV (7). None of the eight nucleosides tested appeared to reverse the antiviral activity of the two compounds (data not shown). Addition of the nucleo-
The antiviral activities of B-220 and PFA were tested on two CMV strains (Table 4). One strain, V2828, exhibited a decreased susceptibility to PFA. A 50% reduction in CMV protein production in HL cells by CMV Ad.169 was obtained with 2 μM B-220; 5 μM was needed to inhibit strain V2828. The corresponding values for PFA were 30 and 400 μM. PFA inhibited CMV late antigens as shown by immunofluorescence but showed no effect against formation of early antigens as shown by ACIF. B-220 inhibited formation of both early and late CMV antigens. B-220, on the other hand, was found to be toxic to HL cells at a concentration of 15 μM, whereas PFA was nontoxic at 800 μM.

To study dependence on multiplicity of infection, B-220 and PFA were tested in HL cells infected with CMV at various multiplicities of infection (Table 5). The results indicated that the concentrations of both B-220 and PFA must be increased to obtain 50% viral reduction with a higher virus dose.

**Inhibitory activity of PFA and B-220 on HSV-1, CMV, and VZV DNA polymerases.** HSV-1 polymerase activity was inhibited in vitro by 50% with 35 μM B-220 (Table 6), but less than 2 μM B-220 was required for a 50% reduction of HSV-1 viral antigen in tissue culture (Table 2). B-220 also exhibited low activity against CMV DNA polymerase. A 50% inhibition of CMV polymerase was obtained with about 100 μM B-220 (Table 6), whereas only a 2- to 5-μM concentration was needed to inhibit CMV viral protein production in cell culture (Table 4). CMV DNA polymerase was inhib-

| TABLE 3. Anti-HSV-1 activity of three antiviral compounds in three cell types as measured by ELISA |
|---------------------------------|-----------------|-----------------|-----------------|
| **Compound** | **IC₅₀ (μM)** in given cell type | **E.J.** | **A549** | **RK-13** |
|-----------------|-----------------|-----------------|-----------------|
| B-220 | 1.51 | 2.19 | 4.17 |
| B-300 | 3.24 | 3.80 | 12.3 |
| B-301 | >100 | >30 | >30 |

**TABLE 4. Activity of B-220 against two CMV strains in HL cells**

| **Compound** | **IC₅₀ (μM)** | **Concn (μM)** required for cellular cytotoxicity*a |
|-----------------|-----------------|-----------------|-----------------|
| **CMV Ad.169** | **CMV V2828** | **ELISA** | **ACIF** | **IF** |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| B-220 | 2 | 4 | 3 | 5 | 4,5 | 5 | 15 |
| PFA | 30 | >400 | 80 | 400 | >400 | 250 | >800 |

*a Live cells were quantified by trypan blue staining.
* IF, Indirect immunofluorescence.
TABLE 5. Inhibitory activity of B-220 and PFA on CMV DNA synthesis as measured by hybridization

| CMV strain | Compound | IC_{50} (μM) at multiplicity of infection of: |
|------------|----------|---------------------------------------------|
|            |          | 4.0                                         |
|            |          | 0.5                                         |
| Ad.169     | B-220    | 1                                           |
|            | PFA      | 25                                          |
|            | B-220    | 3.5                                         |
| V4078      | PFA      | 80                                          |

Addition of PFA at 0.4 μM, whereas about 100 μM was needed in cell culture. Addition of 80 μM B-220 inhibited the VZV polymerase reaction by 50%, but only 0.1 to 0.3 μM (depending on the amount of polymerase) PFA displayed similar activity.

Addition of 1 to 10 μM B-220 did not increase the inhibitory activity of PFA, and addition of 0.01 μM PFA did not increase the inhibitory activity of B-220 (Table 6). A PFA concentration of 0.1 μM increased the activity of B-220 only slightly. A combination of PFA and B-220 did not significantly alter the direct effect of either drug on the level of polymerase. These findings indicate a direct effect of B-220 on viral DNA. PFA is known to interfere with the viral polymerase reaction (4).

DISCUSSION

B-220 at concentrations of 1 to 5 μM showed antiviral activity against HSV-1, CMV, and VZV (Table 7). The ratio of antiviral effect to cellular cytotoxicity ranged between 3 and 15 among the various experiments. The range of ratios could be explained both by differences in susceptibility among cell lines to a direct effect of B-220 and by the effect of multiplicity of infection (9) on the activity of B-220. The activity of B-220 against HSV-1 was better than that of any of the other structural analogs tested. Ellipticine itself did not show any activity against HSV-1, possibly because of its low solubility. The results of these experiments in vivo, 25 mg of B-220 per kg of body weight given intravenously once daily for 4 weeks to rats did not produce signs of toxicity. The levels in blood in this study reached 20 to 80 μM, well above the levels producing antiviral effects in vitro (T. Malmfors, personal communication). In single-dose therapy in rats, a B-220 concentration of 80 mg/kg given intravenously or more than 800 mg/kg given orally was needed to obtain 50% lethality (Malmfors, personal communication). These results suggest that the drug has little toxicity in vivo, in contrast to results obtained in cell culture.

TABLE 6. Inhibitory effect of B-220 and PFA on viral polymerases

| Compound | CMV DNA | VZV DNA | HSV-1 DNA | Calf thymus cell DNA |
|----------|---------|---------|-----------|----------------------|
| B-220    | >100    | 80      | 35        | 10                   |
| PFA      | 0.4     | 0.2     | 0.3       | 100                  |
| B-220 (in combination with 0.01 μM PFA) | >100 | 75 | 33 | 6 |
| PFA (in combination with 1 μM B-220) | 0.4 | 0.2 | 0.3 | 100 |

* Inhibition of Escherichia coli DNA polymerase α resulted in an IC_{50} of B-220 of 800 μM.

TABLE 7. Ranges of susceptibility of herpesviruses to B-220 and control compounds in HL cells

| Cell culture | IC_{50} (μM)* |
|--------------|---------------|
| HSV-1        | B-220         | PFA         | ACV         | 2HM-HBG*     |
|              | 1.5           | 10-150      | 0.1-2.5     | 1.0          |
| CMV          | 0.5-3         | >800        | >800        | >800         |
| CMV early antigen | 0.5-3      | >800        | >800        | >800         |
| VZV          | 0.5-3         | 150-300     | 6.5-20      | 0.4-0.7      |
| HL           | 10-30         | >800        | >800        | >800         |

* Data from this study and references 1, 4, 9, and 15.
* 2HM-HBG, (RS)-9-(4-hydroxy-2-(hydroxymethyl)butyl)guanine.
* Results determined by cytotoxicity.

The antiviral effect of B-220 varied with the cell culture system used for infection. The dependence of antiviral drug activity on the host cell is a well-known phenomenon that has been shown previously for antiviral nucleoside analogs (8, 9). Addition of extracellular naturally occurring nucleosides did not affect the antiviral activity of B-220, which indicates that interaction between B-220 and DNA precursor enzymes or metabolites is not likely. B-220 also does not appear to act in a competitive way as a nucleoside analog.

B-220 inhibited viral polymerases by 50% at concentrations of 35 to 100 μM, exhibiting greater activity in virus-infected cell cultures than on purified polymerases. The opposite is true for enzyme inhibitors such as PFA, ACV, and (RS)-9-(4-hydroxy-2-(hydroxymethyl)butyl)guanine (1, 3, 4; present study). A combination of B-220 and PFA neither increased nor decreased the activity of either drug directly in cell culture or on polymerases. Since the two molecules were combined in a ratio of 1:1, this finding confirms the independence of each compound.

HSV-1 strains resistant to PFA have been shown to exhibit increased susceptibility to aphidicolin (2). We tested whether this collateral susceptibility could be important with respect to B-220. We could not detect any increased susceptibility to B-220 of two CMV strains that had low susceptibility to PFA. On the other hand, we did not see any interference of CMV inhibition when B-220 and PFA were assayed together. This finding also indicates that the actions of these two compounds are independent.

B-220, in contrast to PFA and ACV, inhibited production of both early and late CMV antigens. This finding indicates that viral DNA, even if present, may not be translated. Pyrophosphate or nucleoside analogs do not inhibit translation of early viral antigens (14a, 15; present study). Therefore, B-220 appears to inhibit viral multiplication at an early point in the virus cycle. Hybridization studies showed that CMV DNA synthesis was inhibited by B-220. B-220 is structurally related to ellipticine and quinacrine, compounds known to intercalate into the DNA helix (11). It is therefore likely that intercalation into input or replicating viral DNA contributes to the antiviral activity of B-220.

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