HBB [2-(α-hydroxybenzyl)-benzimidazole] and guanidine are potent inhibitors of picornavirus replication. Among other evidence, limited cross-resistance and a synergistic effect of both inhibitors suggest similar but not identical mechanisms of antiviral action. Echovirus-9 variants resistant to each of these drugs were characterized and sequenced. Complete resistance to HBB or guanidine was shown to be due to single but different point mutations in the non-structural protein 2C. Protein 2C was expressed as GST fusion and His-tagged proteins for the wild-type and various mutants. Although three mutations were located in or near conserved NTP binding motifs, NTPase activity was not altered in the presence of HBB or guanidine.

Introduction

It is well established that 2-(α-hydroxybenzyl)-benzimidazole (HBB) and guanidine selectively inhibit the replication of certain entroviruses (Caliguiri & Tamm, 1973; Eggers, 1982). Neither of these substances affects processes like adsorption, uncoating or viral protein synthesis. However, it has been known for decades that they block viral RNA synthesis (Eggers & Tamm, 1963a; Eggers, 1982).

Many investigations on poliovirus-1 have revealed that guanidine targets the non-structural protein 2C (Baltera & Tershak, 1989; Pincus et al., 1986; Pincus & Wimmer, 1986) and recently, the determinant for dependence on HBB was found to be located within the 2C region of echovirus-9 (E9) (Hadaschik et al., 1999). Therefore, inhibitors like HBB or guanidine may serve as important tools to extend our knowledge of protein 2C and, in consequence, picornavirus replication.

Protein 2C is one of the most conserved polyproteins of picornaviruses (Argos et al., 1984). However, the function of 2C is still not well understood and seems to be complex (Rueckert, 1996; Wimmer et al., 1993). It has been shown that this protein plays an important role in RNA replication, and the presence of 2C in enterovirus replication complexes comprised of membrane particles, viral RNA and other virus-encoded proteins has been demonstrated (Bienz et al., 1990). Functions like membrane binding, protein–protein interactions, NTPase activity and RNA binding have been revealed for poliovirus 2C by different in vitro techniques (Cuconati et al., 1998; Echeverri & Dasgupta, 1995; Mizayen & Wimmer, 1994; Pfister & Wimmer, 1999; Rodriguez & Carrasco, 1993, 1995). NTPase activity was also observed in E9 (Klein et al., 1999).

In this report, resistance to HBB and guanidine was defined at the genetic level, and the influence of these antiviral substances on known functions of E9 2C was investigated.

Methods

- **Virus, replication inhibitors and cells.** E9 strain Barty (E9B), originally isolated in Cincinnati, Ohio (Eggers & Sabin, 1959), was passaged as described (Zimmermann et al., 1996). 2-(α-Hydroxybenzyl)-benzimidazole (HBB) was synthesized by Hoechst and guanidine hydrochloride was obtained from Sigma. For cell culture experiments and virus propagation, green monkey kidney (GMK) monolayer cultures were used (Eggers, 1977). Virus titres were determined by plaque assay.

- **Sensitivity assay.** GMK monolayers were infected with the respective virus and the virus-induced cell damage (CPE) was estimated by microscopic examination (Eggers & Tamm, 1961). The average of at least two independent values, differing by not more than 50%, was taken. Infecting virus doses were standardized to approximately 100 TCID50 values (median 50% tissue culture infective doses).

- **Northern blot analysis.** Total RNA of single-cycle infected GMK cells was isolated using the guanidium thiocyanate protocol (Ausubel et
After spectrophotometric measurement of the total RNA concentrations, 10 µg portions were subjected to denaturing gel electrophoresis (1% polyacrylamide), and blotted onto an uncharged nylon membrane (Amersham) using standard techniques (Ausubel et al., 1994). In vitro-transcribed full-length echovirus RNAs of negative and positive polarity were included as positive controls. Fragment sizes were determined by an RNA mass marker stained separately with ethidium bromide.

After KpnI digestion of the E9B full-length clone rE9B-fl.20 (Zimmermann et al., 1996), the 353 bp fragment comprising nucleotides 2844–3196 was cloned into a KpnI-linearized pT7-α-18 vector (BRL), transcribed in vitro and purified according to standard protocols (Ausubel et al. 1994). These uniformly [32P]CTP-labelled RNAs of both orientations were used as probes in the Northern blotting experiments. For an internal standard, a labelled RNA transcript from a mouse β-actin cDNA clone (Stratagene) was prepared in the same way. Finally, the hybridized membranes were autoradiographed.

**NTPase assay.** Echovirus 2C wild-type and mutants were purified as GST fusion proteins and the ATPase reaction was carried out as previously described (Klein et al., 1999). In brief, approximately 1 µg of protein was incubated for 60 min at 37 °C in reaction buffer (20 mM HEPES–KOH (pH 7–9), 5 mM MgCl₂, 2.5 mM DTT, 0.1% BSA, 0.05% Triton X-100, 50 µM ATP and 10 µCi/ml [γ-32P]ATP (3000 Ci/mmol; Amersham)). For detection, one tenth of the reaction volume was subjected to TLC followed by autoradiography.

**Non-denaturing purification of His-tagged 2C.** The coding region of echovirus 2C was amplified by PCR using the primers ATG GCT GAG CTC GAG AGT AAT GGC TGG CTC AAG (positions 4081–4113) and GTA GAC CTC GAG TTA TGG GAA CAG AGC TTC AGT AAG (positions 5096–5061), which contain manually inserted XhoI restriction sites (in bold). After digestion with XhoI, the PCR product was cloned into a XhoI-linearized pET-14b (Novagen) expression vector. Proper orientation and ligation were controlled by sequence analysis, and the plasmid was transformed into E. coli BL21 (DE3). Expression of His–2C in LB medium was started after induction with 1 mM IPTG at an A600 of 1. After an incubation time of 2 h at 25 °C, the bacteria were harvested and resuspended in lysis buffer [0.5 M NaCl, 20 mM Tris–HCl (pH 7–9), 10 mM imidazole, 2 mM β-mercaptoethanol, 0.1% PMSF, 10% glycerol, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, and 0.2% each of Tween 20, Nonidet P-40 and Triton X-100]. Cells were lysed by three freeze–thaw cycles followed by mild sonication. Further purification steps were performed by Ni²⁺ affinity chromatography according to the manufacturer’s recommendations (Qiagen). After step-wise washing and elution, 2C-containing fractions were analysed by SDS–PAGE, concentrated with spin columns (Amicon), and dialysed against 20 mM HEPES–KOH (pH 7–9), 100 mM KCl, 5 mM DTT and 25% glycerol.

**Results**

**Synergism of HBB and guanidine.**

Sensitivity assays using different concentrations of HBB or guanidine separately as well as in combination were performed. Concentrations of 30 µM HBB or 0.5 mM guanidine did not inhibit multiplication of E9B significantly (Fig. 1a, b). When half of the respective concentrations of each drug were used together, virus multiplication was delayed for more than 2 days (Fig. 1c). This strongly suggests synergism based on...
Resistant viruses were plaque-purified in the presence of one passage without an antiviral agent was performed. Finally, dependent viruses also selected under the chosen conditions, passages with $1 \text{M HBB}$ turned out to be weak (Fig. 2). Guanidine levels but, in contrast, the resistance of E9-G.res to that the multiplication of E9-H.res is possible even at high inhibitors used for their selection. (a) Replication of E9-H.res and E9-G.res in the presence of inhibitors used for their selection. (b) Cross-resistance of E9-H.res and E9-G.res against guanidine and HBB, respectively.

Cross-resistance was investigated and it could be shown that the multiplication of E9-H.res is possible even at high guanidine levels but, in contrast, the resistance of E9-G.res to HBB turned out to be weak (Fig. 2 b).

Cloning and sequencing of E9-H.res and E9-G.res

Viral RNA of E9-H.res was prepared and transcribed and the longest fragment [position 3893 to the 3’ poly(A) tail] was cloned into the infectious full-length wild-type clone rE9B-fl.20 (Zimmermann et al., 1996), giving rise to the recombinant clone rE9-H.res (Fig. 3). Sequence comparison of the HBB-resistant cDNA clones and the wild-type clone revealed amino acid exchanges: $A_{1773}^C$ and $C_{1782}^U$. Both mutations are located in the coding region of 2C (Table 1).

Since the mutations responsible for the guanidine resistance of E9B are also supposed to be located within the 2C gene, viral RNA extending from position 3226 to 5474 was amplified by RT–PCR. The resulting DNA was cloned into the rE9B-fl.20 plasmid giving rise to the construct rE9-G.res (Fig. 3). Sequence analysis also revealed two mutations: $A_{1228}^G$ and $G_{4192}^A$ (Table 1).

Influence of HBB and guanidine on echovirus protein 2C

After transcription of the plasmids rE9-H.res and rE9-G.res in vitro, transfection experiments were performed using the DEAE method (Ausubel et al., 1994), and the resulting viruses were propagated in GMK cells. Sensitivity assays of these recombinants revealed a resistant phenotype as found for the original isolates, E9-H.res and E9-G.res (Fig. 4a). In both cases, virus replication was possible even at high inhibitor concentrations, indicating that the detected mutations in the 2C gene are relevant for drug resistance.

In order to characterize the significance of each mutation, single nucleotide exchanges were introduced separately into rE9B-fl.20 by site-directed mutagenesis. Sensitivity assays of the resulting viruses revealed that the recombinants bearing mutation $A_{1773}^C$ (rE9-H.res and rE9-A$_{1773}^C$) exhibited the same phenotype as E9-H.res (Fig. 4b). Hence, this nucleotide exchange appears to be the determinant for HBB resistance. However, the second mutation found in the HBB-resistant isolate, C$_{1782}^U$, turned out to introduce dependence on HBB or alternatively on guanidine (Fig. 4c). Analogous experiments with the mutations of the guanidine-resistant isolates revealed that mutation $G_{4192}^A$ leads to a complete guanidine-resistant phenotype and mutation $A_{1228}^G$ causes drug resistance with an obvious tendency to guanidine-dependence (Fig. 4b, c).

Like the originally isolated HBB-resistant variant, the recombinant mutants derived from E9-H.res were able to multiply well in the presence of guanidine (Fig. 4). On the other hand, the mutants derived from isolate E9-G.res were only partially resistant to HBB.

Effect of HBB on viral RNA replication

To investigate whether mutations in protein 2C directly influence RNA replication, both positive- and negative-strand RNA synthesis by the virus variants were analysed using single-cycle infection experiments followed by Northern blotting. When using the HBB-sensitive E9 wild-type, no positive-strand RNA was detectable in the presence of HBB.
Table 1. Mutations of HBB-resistant (rE9-H.res) and guanidine-resistant (rE9-G.res) clones compared to the wild-type E9 2C region

<table>
<thead>
<tr>
<th>Variant</th>
<th>Mutation*</th>
<th>nt position†</th>
<th>Sequence divergence</th>
<th>aa position‡</th>
<th>aa divergence</th>
<th>Affected functional domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9-H.res</td>
<td>a</td>
<td>4775</td>
<td>A → C</td>
<td>227</td>
<td>I → L</td>
<td>NTPase motif ‘C’</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>4782</td>
<td>C → U</td>
<td>229</td>
<td>A → V</td>
<td>NTPase motif ‘C’</td>
</tr>
<tr>
<td>E9-G.res</td>
<td>c</td>
<td>4286</td>
<td>A → G</td>
<td>64</td>
<td>E → G</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>4492</td>
<td>G → A</td>
<td>133</td>
<td>A → T</td>
<td>NTPase motif ‘A’</td>
</tr>
</tbody>
</table>

* See Fig. 3.
† Positions relative to whole virus genome.
‡ Positions relative to protein 2C.

Fig. 4. Sensitivity assays of the recombinant viruses containing both mutations (a) or single mutations (b and c). ○, No inhibitor; ●, 73 µM HBB; ■, 146 µM HBB; ▲, 220 µM HBB; △, 1 mM guanidine; ▽, 2 mM guanidine. See text for nomenclature of constructs.

after a replication time of 4 or 6 h (Fig. 5a). Also, in contrast to the uninhibited samples, negative-strand RNA could not be detected at these time-points (Fig. 5b). In an analogous study, the replication assay was carried out using the recombinant viruses. The resistant mutants rE9-H.res and rE9-A4775C were able to replicate the RNA in the absence and presence of HBB,
Influence of HBB and guanidine on echovirus protein 2C

Recently, it has been shown that E9 protein 2C possesses ATPase/GTPase activity (Klein et al., 1999). With one exception, A<sub>1298</sub>G, the mutations of the HBB- and guanidine-resistant E9 variants are located within or near conserved protein domains of 2C which are proposed to be involved in NTP binding or splitting (Table 1). Therefore, the influence of HBB and guanidine on the NTPase activity of several GST–2C fusion protein constructs (GST2C-wt, GST2C-H.res, GST2C-A<sub>1770</sub>C, GST2C-C<sub>1792</sub>U and GST2C-G.res) was tested. In the presence of HBB, however, the ATPase activity of wild-type 2C, GST2C-H.res, GST2C-A<sub>1770</sub>C, and GST2C-C<sub>1792</sub>U was not altered, even in the presence of 1 mM HBB (Fig. 6). Also, guanidine did not abolish ATPase activity at physiologically relevant concentrations.

Besides the GST–2C fusion proteins, His-tagged proteins were constructed to minimize the possibility of unspecific reactions. Therefore, echovirus 2C was cloned into a pET-14b vector. After expression in E. coli BL21, the His-tagged proteins were purified to homogeneity by affinity chromatography under non-denaturing conditions. The His–2C proteins exhibited NTPase activity, and they also remained unaffected by HBB or guanidine (data not shown).

**Discussion**

In this study, we present data showing that the antiviral substance HBB inhibits at least positive-strand RNA synthesis of E9. In addition, an increase in negative strands could not be detected by Northern blotting techniques. Additionally, HBB and guanidine do not affect the NTPase activity of the non-structural protein 2C.

We have demonstrated that the picornavirus replication inhibitors HBB and guanidine seem to differ in terms of their precise mechanism of antiviral action. The limited or, more precisely, unidirectional cross-resistance of the two drugs and the fact that HBB and guanidine exhibit a synergistic action allow us to propose that the inhibitors act in a similar, but not identical way.

To elucidate the mechanisms of antiviral action of both substances at the molecular level, a panel of experiments was performed using drug-resistant E9B variants. For poliovirus, it is known that mutations crucial for resistance against or dependence on picornavirus replication inhibitors map to the coding region of 2C (Pincus et al., 1986; Pincus & Wimmer, 1986). In addition, previous work in our laboratory revealed that the only determinant for HBB dependence of E9B is located within the 2C gene (Hadaschik et al., 1999). Consequently, we focused our interest on this region of the viral genome. Indeed, two mutations were detected in the 2C coding region of each resistant variant and two of them are sufficient to induce a phenotype identical to the original resistant isolates. For guanidine-resistant or -dependent poliovirus, it has been revealed that most of the responsible amino acid substitutions are located at position 179 or 187, respectively (Baltera & Tershak, 1989; Tolskaya et al., 1994). However, in the case of E9, the crucial amino acid exchanges conferring resistance are located at different sites, i.e. position 133 (guanidine) and 227 (HBB), and may suggest different ways of drug inhibition (Table 1).
The exchange $C_{4782}U$ leads to an HBB-dependent phenotype (Fig. 4). It is noteworthy that the identical exchange has been determined in an independent project with the aim of isolating an HBB-dependent E9B variant (Hadaschik et al., 1999). Whether this constitutes mere coincidence — an unlikely possibility — or the only molecular mechanism for HBB dependence can only be elucidated by investigation of a larger number of dependent E9B isolates.

Three of the four mutations characterized in this project are located in the direct vicinity of two of three predicted NTP binding and splitting domains of 2C (Table 1). The NTPase activity of these domains was proven in previous work (Klein et al., 1999). However, 2C fusion proteins of both E9B wild-type and resistant mutants did not exhibit altered NTPase activity in the absence or presence of physiologically active inhibitor concentrations (Fig. 6). The weak inhibitory effect of 20 mM guanidine is probably due to the initiation of protein denaturation. Therefore, the NTPase activity of E9 2C does not appear to be a target for HBB or guanidine (data not shown). Taken together, since neither the NTPase nor the RNA binding capacity of 2C could be affected by the inhibitors in vitro, our results support the conjecture that there may be an additional function of 2C acted upon by the drugs. A helicase activity as well as involvement in packaging have been discussed for picornavirus (Gorbalenya et al., 1990; Kadaré & Haenni, 1997; Li & Baltimore, 1990; Vance et al., 1997). Because inhibition of RNA synthesis of E9 was proven at least for HBB (Fig. 5a, b), it is more likely that the helicase function is affected by the antiviral drugs, if such an activity is actually true for protein 2C.

Preliminary experiments allow the assumption that echovirus 2C binds ssRNA unspecifically, and again this 2C function does not seem to be influenced by HBB or guanidine (data not shown). Taken together, since neither the NTPase nor the RNA binding capacity of 2C could be affected by the inhibitors in vitro, our results support the conjecture that there may be an additional function of 2C acted upon by the drugs. A helicase activity as well as involvement in packaging have been discussed for poliovirus (Gorbalenya et al., 1990; Kadaré & Haenni, 1997; Li & Baltimore, 1990; Vance et al., 1997). Because inhibition of RNA synthesis of E9 was proven at least for HBB (Fig. 5a, b), it is more likely that the helicase function is affected by the antiviral drugs, if such an activity is actually true for protein 2C.

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