Background: Ribavirin is a broad-spectrum antiviral agent that derives some of its activity from inhibition of cellular inosine monophosphate dehydrogenase (IMPDH), resulting in lower guanosine triphosphate (GTP) levels. Here we report the biological activities of three ribavirin analogues.

Methods: Antiviral activities of test compounds were performed by in vitro cytopathic effect inhibition assays against influenza A (H1N1, H3N2 and H5N1), influenza B, measles, parainfluenza type 3 (PIV-3) and respiratory syncytial viruses. Compounds were modelled into the ribavirin 5′-monophosphate binding site of the crystallographic structure of the human type II IMPDH (hIMPDH2) ternary complex. Effects of compounds on intracellular GTP levels were performed by strong anion exchange HPLC analysis.

Results: Of the three compounds evaluated, the 5-ethynyl nucleoside (ETCAR) exhibited virus-inhibitory activities (at 1.2–20 μM, depending upon the virus) against most of the viruses, except for weak activity against PIV-3 (62 μM). Antiviral activity of ETCAR was similar to ribavirin; however, cytotoxicity of ETCAR was greater than ribavirin. Replacing the 5-ethynyl group with a 5-propynyl or bromo substituent (BrCAR) considerably reduced antiviral activity. Computational studies of ternary complexes of hIMPDH2 enzyme with 5′-monophosphates of the compounds helped rationalize the observed differences in biological activity. All compounds suppressed GTP levels in cells; additionally, BrCAR suppressed adenosine triphosphate and elevated uridine triphosphate levels.

Conclusions: Three compounds related to ribavirin inhibited IMPDH and had weak to moderate antiviral activity. Cytotoxicity adversely affected the antiviral selectivity of ETCAR. As with ribavirin, reduction in intracellular GTP may play a role in virus inhibition.

Introduction

For many years the antiviral agent ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, 1; Figure 1) [1,2], has been used clinically to treat respiratory syncytial virus (RSV) infections in children [3]. More recently, the combination of ribavirin and interferon-α has become a mainstay in the treatment of HCV infections in humans [4]. The mode of action of 1 against different pathogenic RNA viruses has been a complex issue, since it may involve several mechanisms that contribute to the overall effect, depending on virus species [5,6]. Inosine monophosphate dehydrogenase (IMPDH) inhibition by ribavirin 5′-monophosphate (RMP; 2) was the first proposed mechanism [7] and proved essential for antiviral activity against flaviviruses [8], paramyxoviruses [8] and orthopoxviruses [9]. Being the substrate analogue, RMP competitively inhibits IMPDH, resulting in low levels of intracellular guanosine triphosphate (GTP) that no longer sustain viral replication. The major metabolite of 1, ribavirin 5′-triphosphate may inhibit GTP-dependent RNA capping enzymes causing interference in the viral messenger RNA maturation process [10]. Moreover, ribavirin 5′-triphosphate interacts with viral RNA-dependent RNA polymerases of different viral species [6,11]. After incorporation into viral RNA, ribavirin can either terminate the synthesis or induce mutations. Accumulation of mutations in
viral genomic RNA may result in error catastrophe for a virus. This distinctive mechanism was postulated to explain antiviral activity of ribavirin against poliovirus [12]. To date, mutagenic activity of the drug has been found to be responsible for error-prone replication of other viruses such as GB virus B [13], HCV [14], Hantaan virus [15] and West Nile virus [16]. Recently, studies to elucidate cell-based resistance after continuous treatment with this antiviral agent have shown that, depending on its intracellular metabolism, ribavirin inhibits replication of the same virus via different mechanisms in different cell types [17].

Along with its application as a drug, ribavirin has become a lead compound for many other nucleoside analogues. One of them, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR; 3), demonstrates broad-spectrum antiviral activity against DNA and RNA viruses comparable to that of ribavirin, although the former compound exceeds the latter in potency by 10- to 100-fold [18]. When metabolized to 5′-monophosphate (EICARMP; 4), it acts primarily as a mechanism-based inhibitor of IMPDH [19], covalently binding to a cysteine residue of the enzyme active site [20]. It may be further phosphorylated to 5′-O-triphosphate. Due to its toxicity, EICAR has no longer been investigated as a drug candidate for treatment of viral infections in humans. Nevertheless, EICAR has shown efficacy against animal viruses such as infectious pancreatic necrosis virus (the pathogen of many marine species) [21] and canine distemper virus.

Figure 1. Chemical structures of triazole compounds related to ribavirin and ribavirin 5′-monophosphate
Antiviral Chemistry & Chemotherapy 23.4

Materials and methods

Chemistry

General methods and techniques were applied to the synthesis of ETCAR (10) and ProTCAR (11) according to prior publications [27, 28]. Specifics for the synthesis of 5-bromo-1-[β-D-ribofuranosyl]-1H-[1,2,3]triazole-4-carboxamide (BrCAR; 12) are given below. Compound 12 and its intermediates were characterized by 1H and 13C NMR spectra recorded on a Bruker 400 spectrometer (Bruker Optics, Poznan, Poland) operating at 400 MHz and 100.6 MHz, respectively. Mass spectra were recorded using Bruker microTOF-q mass spectrometer (Bruker Optics). Ribavirin and 3-deaza-guanine were obtained from the former ICN Pharmaceuticals (Costa Mesa, CA, USA).

Synthesis of 5-bromo-1-[β-D-ribofuranosyl]-1H-[1,2,3]triazole-4-carboxamide, 12

To a suspension of 1-(5-O-acetyl-2,3-O-isopropylidene-β-D-ribofuranosyl)-5-amino-1H-[1,2,3]triazole-4-carboxylic acid amide [29] (790 mg, 2.31 mmol) dissolved in vacuo over P2O5 at 90°C, for 4 h in bromoform (34.4 g, 136 mmol) was added isooamyl nitrite (949 mg, 8.1 mmol). The mixture was stirred at 100°C for 2 h, then it was applied onto a silica gel column. The column was eluted with CH2Cl2 (in order to collect the bromoform) followed by CH3Cl-MeOH (95:5) to give crude product. It was next re-chromatographed using ethyl acetate/hexane (1:1→2:1) to afford 537 mg (55% yield) of 5-bromo-1-(5-O-acetyl-2,3-O-isopropylidene-β-D-ribofuranosyl)-1H-[1,2,3]triazole-4-carboxylic acid amide. 1H NMR (DMsol-d6) δ 7.96 and 7.63 (2x, 2H, CO-NH2), 5.66 (d, J12,3 = 4.0 Hz, 1H, 1′-H), 5.66 (d, J1OH,3OH = 5.6 Hz, 1H, 2′-OH), 5.31 (d, J12,5OH = 6.0 Hz, 1H, 3′-OH), 4.78 and 4.75 (t overlap, dd, J12,5 = 5.6 Hz, 2H, 5′-OH, 2′-H), 4.28 (dd, J12,2 = 4.8 Hz, J12,5OH = 6.0 Hz, 1H, 1′-H), 3.99 (dd, J12,5 = 7.2 Hz, J12,2 = 5.6 Hz, 1H, 4′-H), 3.53 and 3.41 (2x, 2H, 3′-H). 13C NMR (DMso-d6) δ 161.06 (CO-NH2), 139.29 (C-4′), 115.30 (C-5), 90.34 (C-1′), 86.37 (C-4′), 73.58 (C-2′), 70.53 (C-3′), 61.58 (C-5′). HRMS [M+Na]+ calcd for C13H13BrN4NaO6: 385.0118; found: 385.0126.

Isopropylidene blockade was cleaved with an ice-cooled 80% aqueous trifluoroacetic acid (4 ml/1 mmol of substrate). After the reaction was complete (0°C, 2 h), the solution was co-evaporated several times with EtOH, then with anhydrous toluene. The oily residue was dissolved in EtOAc/MeOH (10:1) and evaporated slowly to enforce precipitation of the crude product. It was then separated and dried in vacuo to a white powder (mp 126°C, 80% yield). 1H NMR (DMso-d6) δ 7.96 and 7.63 (2x, 2H, CO-NH2), 5.65 (d, J12,3 = 4.0 Hz, 1H, 1′-H), 5.66 (d, J12,5OH = 5.6 Hz, 1H, 2′-OH), 5.31 (d, J12,5OH = 6.0 Hz, 1H, 3′-OH), 4.78 and 4.75 (t overlap, dd, J12,5 = 5.6 Hz, 2H, 5′-OH, 2′-H), 4.28 (dd, J12,2 = 4.8 Hz, J12,5OH = 6.0 Hz, 1H, 1′-H), 3.99 (dd, J12,5 = 7.2 Hz, J12,2 = 5.6 Hz, 1H, 4′-H), 3.53 and 3.41 (2x, 2H, 3′-H). 13C NMR (DMso-d6) δ 160.45 (CO-NH2), 139.52 (C-4′), 115.16 (C-5), 112.62 (CH3)2C, 91.57 (C-1′), 89.04 (C-4′), 83.19 (C-2′), 81.78 (C-3′), 60.86 (C-5′), 26.65, 24.95 (CH3)2C. HRMS [M+Na]+ calcd for C13H13BrN4NaO6: 385.0118; found: 385.0126.

Decaytation of the compound (435 mg, 1.04 mmol) was performed with sodium methoxide (83 mmol) in anhydrous methanol (13 ml) at 0°C. After the reaction was complete (3 h), neutralization with Dowex H+ (100 mg) prewashed with anhydrous methanol followed. The filtrate was evaporated and the residual oily product was dried in vacuo to give 370 mg (98% yield) of 5-bromo-1-(2,3-O-isopropylidene-β-D-ribofuranosyl)-1H-[1,2,3]triazole-4-carboxylic acid amide. 1H NMR (DMso-d6) δ 7.97 and 7.65 (2x, 2H, CO-NH2), 6.17 (s, 1H, 1′-H), 5.62 (d, J12,3 = 6.0 Hz, 1H, 2′-H), 4.98 (dd, J12,3 = 6.0 Hz, J12,5OH = 1.6 Hz, 1H, 3′-H). 4.91 (t, J12,5OH = 5.6 Hz, 1H, 5′-OH), 4.21 (dt, J12,3 = 6.0 Hz, J12,5OH = 1.6 Hz, 1H, 4′-H), 3.18 (m, 2H, 5′-H), 1.52 and 1.36 (2x, 6H, 2×CH3). 13C NMR (DMso-d6) δ 160.45 (CO-NH2), 139.52 (C-4′), 115.16 (C-5), 112.62 (CH3)2C, 91.57 (C-1′), 89.04 (C-4′), 83.19 (C-2′), 81.78 (C-3′), 60.86 (C-5′), 26.65, 24.95 (CH3)2C. HRMS [M+Na]+ calcd for C13H13BrN4NaO6: 385.0118; found: 385.0126.

In vivo antiviral evaluations

Viruses and cells

Influenza A/California/07/2009 (H1N1), A/Perth/16/2009 (H3N2) and B/Florida/4/2006 viruses were obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA). Influenza A/Duck/
MN/1525/81 (H5N1), a low pathogenic avian virus isolated in the United States, was kindly provided by Robert Webster (St Jude Children’s Research Hospital, Memphis, TN, USA). Experiments with the above influenza virus strains were carried out in Madin-Darby canine kidney (MDCK) cells. Parainfluenza type 3 (PIV3) virus strain 14702, RSV strain A, and measles virus strain CC were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Experiments with PIV-3 and RSV were performed in African green monkey kidney (MA-104) cells, whereas measles virus was assayed in the Vero 76 line of African green monkey kidney cells. The cell lines were all obtained from ATCC.

Antiviral assays
Inhibition of viral cytopathic effect assays were performed as previously described using confluent monolayers of cells in 96-well microplates [30], with cytotoxicity determined in parallel in uninfected cells. Trypsin (10 units/ml; Sigma–Aldrich, St Louis, MO, USA) in serum-free medium was required to facilitate the replication and cytopathology of influenza A and B viruses. Assays with the other viruses were performed in medium containing 2% fetal bovine serum. Quantitation of percent cytopathic effect was accomplished by treating cells with 0.011% neutral red dye for 2 h, then determining dye uptake into viable cells [31]. Unincorporated dye was removed from cells by aspiration and rinsing with phosphate-buffered saline, then the absorbed dye was eluted from the cells for 30 min with 0.1 ml of 50% Sörensen’s citrate buffer (pH 4.2)/50% ethanol. The plates were read for optical density determinations at 540 nm with 405 nm reference. Readings were converted to percentages of uninfected control and normalized to the virus control. The 50% virus inhibitory concentration (IC50) values and 50% cytotoxic concentration (CC50) values were determined by linear regression analysis. Selectivity index (SI) values were calculated as CC50/IC50.

Computational methods with IMPDH
Analysed 5'-monophosphates of 1,2,3-triazole nucleosides: 13, 14 or 15 were modelled into the substrate binding site of the solved X-ray crystallographic structure of hIMPDH2 complexed with RMP (compound 2) as substrate and C2-mycophenolic adenine dinucleotide as a cofactor analogue. The crystal structure of hIMPDH2 was taken from the Protein Data Bank: 1NF7 [32]. To our knowledge this crystal structure has never been published in a scientific journal. Modelling was performed with the Accelrys Discovery Studio 3.0 suite of programmes (Accelrys Software, San Diego, CA, USA).

All water molecules were removed and hydrogen atoms were added. While coordinates of all heavy atoms of the enzyme were kept frozen, hydrogen atoms positions were optimized with the use of adopted basis Newton-Raphson minimization algorithm (max steps =500, RMS gradient =0.01, distance-dependent dielectrics as implicit solvent model). Compounds 13, 14 or 15 were initially modelled into the binding site via super-positioning onto the RMP binding site of the hIMPDH2 crystal structure.

For the docking procedure the dock ligands (CDOCKER) protocol, an implementation of the CDOCKER algorithm [33] within the Discovery Studio 3.0 package was used. Random ligand conformations were generated from the initial ligand structure through high temperature molecular dynamics at 700 K. The binding site was defined from the volume of a co-crystallized ligand (RMP). The random conformations were refined by grid-based simulated annealing and a final full force field minimization. In all calculations for proteins and ligands, CHARMM forcefield with MMFF94 partial charge rules was used. To account for protein flexibility, the ligand binding site of each model was further refined by restraint energy minimization.

HPLC analysis of intracellular nucleotide pools
Confluent MDCK cell cultures in 6-well microplates were treated with compounds in serum-free medium for 24 h. The medium was aspirated from each well, followed by precipitation and breaking open of the cells with 3.5% perchloric acid and neutralization of the acid with 1 N KOH/1 M imidazole. The supernates were collected from each well and frozen at -80°C until analysed by HPLC.

HPLC analysis of the samples was conducted as previously published [34]. Briefly, chromatographic separations were performed using an HPLC apparatus (Waters Corp., Milford, MA, USA) fitted with a 10×250 mm column packed with Whatman partisil SAX resin (Phenomenex, Torrance, CA, USA). A linear gradient from 10 mM to 1 M potassium phosphate (Sigma–Aldrich) at pH 5.0 was run over 30 min at 1 ml/min. The 1 M buffer ran an additional 2 min before re-equilibrating the column in low salt buffer. Detection of nucleotides was made at 260 nm with a Waters LC spectrophotometer and the peaks analysed using an integrator (Shimadzu Scientific Instruments, Columbia, MD, USA). Relative peak areas of D282-treated cells were compared to untreated cells and expressed as percentages of control levels.

Results
Synthesis of compounds
Alkynyl-1,2,3-triazole nucleosides 10 and 11 (Figure 1) were prepared by Stille and copper-free Sonogashira reactions, respectively, as described [27,35]. Other authors synthesized 10 according to a standard Sonogashira coupling [36]. Bromotriazole nucleoside 12 (Figure 1) was prepared by diazotization of a protected
5-amino-precursor with organic nitrite in bromoform, followed by a two-step deprotection. Importantly, several groups reported on halogenation to take place at C-5 of 1,4-disubstituted 1,2,3-triazoles during reactions of 5-cuprated 1,2,3-triazole derivatives (key intermediates in CuAAC reactions) with electrophiles: F, Br and Cl [36–40]. In addition, O-protected N-substituted derivative of 12 could be found among compounds prepared to exemplify this new and versatile method [37,38].

In vitro antiviral and cytotoxic activities

Primary in vitro screening tests were conducted against a broad panel of 12 RNA viruses. Further assays allowed us to select compounds 10–12 (Figure 1) for interesting antiviral activity profiles. ETCAR was found to be the most active of the three compounds evaluated, inhibiting both orthomyxo- and paramyxoviruses (Table 1). ProTCAR was only weakly inhibitory to influenza, measles and PIV-3. A greater effect was seen against RSV. BrCAR exhibited its greatest inhibitory effect against influenza B and RSV. Concurrently run cytotoxicity assays demonstrated that ETCAR exhibited greater cytotoxicity than the other compounds. This made its SI values smaller than those of ribavirin, which exhibits less toxicity. Otherwise, the potencies of ETCAR and ribavirin (as demonstrated by IC$_{50}$ values) were quite similar against the various viruses.

Interaction of compounds with IMPDH

The structural resemblances of 10 and 11 to ribavirin and EICAR might imply several plausible mechanisms of antiviral action. First of all, IMPDH inhibition should be considered, assuming activation of nucleosides 10 and 11 to corresponding 5′-O-phosphates 13 and 14 (Figure 1). To gain insight into possible interactions with the enzyme and to correlate observed differences to biological activity of compounds, computational modelling studies on the complexes of hIMPDH2 with substrate analogues 13, 14 or 15, were undertaken as a complementary study.

Compound 13 (ETCARMP) was modelled into the RMP (2) binding site in the crystallographic structure (PDB: 1NF7) of the hIMPDH2 ternary complex. The phosphate moiety and ribose of 13 show consistent overlap with these parts of RMP and form the analogous hydrogen-bonding network with the conserved residues of the IMP binding pocket, as was observed in the crystal structures of IMPDH complexes with IMP, XMP or XMP covalently bound to the key catalytic cysteine residue. The ribose ring hydroxyl groups form a hydrogen-bonding network to Ser68 and Asp364. The phosphate moiety forms bifurcated hydrogen bonds with Gly387, two hydrogen bonds with Ser388 and one with Gly326 (Figure 2A).

The 4,5-disubstituted 1,2,3-triazole ring is well accommodated in the substrate binding site of the hIMPDH2 enzyme and makes three hydrogen bonds with the active site flap: N(2) and N(3) of triazole backbone amide NH group, N(6)H atoms of 4-carboxamide group form a hydrogen bond with Gly415 backbone carbonyl. The heterocyclic N(3) and amide N(6) atoms are on the same side of the triazole moiety. In this configuration N(6) and O(6) atoms are spatially similar to the N(6) and O(6) atoms of adenosine, which can be of great importance for the biological activity of ETCARM (13), because its antiviral activity might be exerted at least in part through its mutagenic activity. This configuration that depends on the rotation of the carboxamide group in 5-amido 1,2,3-triazoles shows lower relative energy compared to biological activity of compounds, computational modelling studies on the complexes of hIMPDH2 with substrate analogues 13, 14 or 15, were undertaken as a complementary study.

<table>
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<tr>
<th>Virus</th>
<th>Cell line</th>
<th>Compound 10 (ETCAR)</th>
<th>Compound 11 (ProTCAR)</th>
<th>Compound 12 (BrCAR)</th>
<th>Positive control</th>
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<tr>
<td></td>
<td></td>
<td>IC$_{50}$ μM</td>
<td>CC$_{50}$ μM</td>
<td>IC$_{50}$ μM</td>
<td>CC$_{50}$ μM</td>
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Viral cytopathic effect inhibition assays were conducted by a neutral red dye uptake method. Ribavirin was the positive control for testing influenza, parainfluenza type 3 (PIV-3) virus, and respiratory syncytial virus (RSV). 3-deazaguanine was the positive control for testing measles. Data for 50% inhibitory concentration (IC$_{50}$) and 50% cytotoxic concentration (CC$_{50}$) are mean (±SD) of 2–3 independent assays. The selectivity index (SI) was calculated as CC$_{50}$/IC$_{50}$.

Antiviral Chemistry & Chemotherapy 23:4

165

Antiviral active ribavirin analogues
than the opposite one (10.891 kJ/mol versus 49.268 kJ/mol) [41] as was found in the crystallographic structure of 5-fluoromethyl-1-β-D-ribofuranosyl-1H-[1–3]triazole-4-carboxamide [28].

By contrast, examination of the X-ray structures of the RMP complexes with IMPDH revealed that in all of them ribavirin (1) is an excellent substrate mimic and its O(7) and N(8) atoms are spatially similar to the O(6) and N(1) atoms of inosine and guanosine [42,43]. This configuration of 3-carboxamide group was also found in two polymorphic ribavirin crystalline forms in the X-ray structure of

Figure 2. Computational modelling and docking of the compound 13 (ETCARMP) into the ribavirin 5′-monophosphate (2) binding site in the crystallographic structure of the hIMPDH2

Crystallographic structure from Protein Data Bank: 1NF7. (A) Schematic representation of the structure and hydrogen-bonding network of ETCARMP in the substrate binding site. (B) Visualization of the substrate binding site in the ternary complex of human type II inosine monophosphate dehydrogenase (hIMPDH2) with NAD+ and ETCARMP. The atoms of NAD+, the catalytic Cys331 and Thr333 residues are highlighted by a coloured space-filling model representation. (C) Superimposition of the dominant poses of docked ETCARMP to the hIMPDH2 substrate binding site. The catalytic Cys331 is highlighted.
1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide [44]. The rotation of ribavirin’s (1) carboxamide group has much lower energy barrier (25.593 kJ/mol versus 20.229 kJ/mol calculated on the level of the CONH$_2$ substituted 1,2,4-triazoles) [41]. In consequence, ribavirin can be incorporated into RNA as a base analogue of either adenine or guanine and it pairs equally well with either uracil or cytosine, inducing mutations in the viral genome [45,46]. One can expect that ETCARMP (13) may act as mutagenic agent as well and base-pair with uracil.

Similar to EICARMP (4), ETCARMP (13) may be a potent irreversible inactivator of the hIMPDH2 enzyme [20]. Because of the anti conformation of the glycosidic bond (γ=245°), the 5′-ethyl substituent lies away from the sugar ring and proximal to the catalytic Cys331 assisted by Thr333. It has been shown that Thr333 residue is completely conserved among IMPDHs [47] and its mutation decreases the rate of hydride transfer, suggesting that Thr333 may influence the reactivity of the catalytic Cys by activating the thiol [48]. The sulfur atom of Cys331 and the electrophilic carbon atom of the 5-ethyl group are separated by 2.6 Å (Figure 2A and 2B). This is an excellent position for a nucleophilic attack and formation of a covalent adduct. Although no stacking interaction between the cofactor nicotinamide ring and the triazole is observed, a distance between the C4 of nicotinamide moiety and the ethynyl group is very short (2.6 Å; Figure 2B). The reactivity of the 5-ethynyl is enhanced by hydrogen-bonds between the triazole moiety and the main chain at residues Met414 and Gly415.

CDOCKER docking results are shown in Figure 2C. One can see that dominant poses of ETCARMP are similarly located at the IMP binding pocket, as it was observed in the crystal structures of IMPDH complexes with IMP, XMP* or XMP covalently bound to the catalytic cysteine residue.

Compound 14 (ProTCARMP), when modelled into the substrate binding site of hIMPDH2 enzyme in the 1NF7 crystallographic structure using the same procedure as for 13, does not have enough manoeuvring room. The methyl of the 5-propynyl substituent comes into collision with Gly326 residue of the active site loop and the nicotinamide portion of NAD$. In the absence of the cofactor, ProTCARMP is located in the substrate binding site similar to ETCARMP, with slight reorientation of the 5-propynyl substituent toward the empty NAD$ site (Figure 3A). In consequence, the electrophilic carbon atom of the 5-propynyl group shifts away from the nucleophilic sulfur atom of Cys331. The glycosidic torsion angle, which describes the relative orientation of the base with respect to the sugar,
is similar to compound 13, in the anti conformation, close to high anti region ($\chi=254^\circ$). It has been shown previously, that the antiviral effectiveness of several analogues of ribavirin is related to their stability in the high anti/anti conformation [49].

Figure 3B presents dominant poses of ProTCARMP obtained in the CDOCKER docking procedure. The orientation of ProTCARMP in the substrate binding site is like ETCARMP with the reorientation of the 5-propynyl group shifting away from the Gly326 residue.

The bulky 5-bromo substituent in compound 15 substantially influences its binding properties. When modelled into the substrate binding site of hIMPDH2 enzyme, 15 does not have enough room and the 5-bromo substituent comes into collision with neighbouring residues (Figure 4). Docking analysis with CDOCKER procedure shows poses with different orientations of the base within the binding pocket (Figure 4B and 4C). The predominant two groups of poses were found, characterized by the glycosidic torsion angle $\chi$ in the region of 173$^\circ$ and 224$^\circ$, corresponding to anti conformation. Our results of antiviral screening of compound 12 showing that it is weakly active are consistent with previous reports of other ribavirin analogues containing bulky substituent in the 5-position [50].

Effects of compounds on intracellular nucleotide pools
The IMPDH assays described above do not directly prove whether treatment of cells with the compounds leads to reductions in intracellular GTP concentrations as a result of inhibition of IMPDH. For a direct demonstration we treated MDCK cells with compounds 10–12 and ribavirin, followed 24 h later by an analysis of intracellular nucleotide pools (Figure 5). Treatment of cells with all compounds resulted in suppression of intracellular GTP levels, but higher extracellular concentrations of compounds 11 and 12 were required to achieve inhibition comparable to compound 10 and ribavirin. Compound 10 had greater inhibitory effects on GTP pools than ribavirin at 3.2 to 100 $\mu$M. Minor inhibitory effects on cytidine triphosphate (CTP) levels occurred at higher concentrations of compound 11 and ribavirin. Compounds 10, 11 and ribavirin had minimal effects on uridine triphosphate (UTP) and adenosine triphosphate (ATP) levels. Compound 12 was most unusual because treated cells exhibited high levels of UTP, some elevations in CTP, and decreases in ATP levels. These results suggest that 12 has more than one cellular activity besides inhibition of IMPDH.

Discussion
In these studies, we demonstrated how small or profound changes at the C-5 position of 1,2,3-triazole-4-carboxamide, a pseudobase isomeric to that of ribavirin, modulate the antiviral activity of nucleosides 10–12. It was determined that the smaller the substituent (ethynyl, 10), the greater the antiviral potency. The presence of the bulky 5-bromo group greatly diminished the antiviral activity of 12. We could rationalize the observed activity of 10 and 11 against orthomyxoviruses enabling their 3′-monophosphates 13 and 14, respectively, to inhibit IMP dehydrogenase in host cells. Assuming that a cellular enzyme, adenosine kinase effectively transforms 10–12 into metabolites 13–15, we modelled each of the latter compounds into the substrate binding site of hIMPDH2 enzyme in the 1NF7 crystallographic structure. Only the least hindered compound 13 fitted perfectly, whereas 14 and 15 located into the site in the absence of NAD$^+$. The docking analysis of 15 showed that the compound preferentially adopted the opposite orientation in the substrate binding side with respect to the compounds 13 and 14, and in consequence would be useless as an inhibitor of the enzyme. On the contrary, the docking analyses of 13 and 14 supported our presumption that both compounds would be inhibitory to the enzyme hIMPDH2, although with different ability.

Results of studying effects of compounds on intracellular nucleotide pools revealed a more complex picture than simple reduction in GTP levels. Compounds 11 and 12 were approximately equally suppressive of GTP, yet compound 12 had additional effects on UTP, CTP and ATP. Inhibition of ATP pools has negative consequences on cell function that may manifest as toxicity, such as in an animal. In cell culture, overt toxicity sometimes is not manifest, particularly if the assays are performed in stationary monolayers as was done here. The two compounds (11 and 12) had similar but not identical antiviral sensitivities. Whether compounds 10, 11 or 12 phosphorylate up to the triphosphate level is not known. Evidence for phosphorylation to the monophosphate level is provided by the fact that treatment of cells results in GTP suppression.

It has been pointed out that a drug combination therapy of influenza virus infections would be feasible to prevent an emergence of new drug-resistant strains of the virus [51]. Antiviral drugs used in combination should exert their activities by different modes of action [52]. Thus, ribavirin or a selection of its analogues could be important components of such combinations.

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Disclosure statement

The authors declare no competing interests.

References


Figure 5. Nucleotide pool changes in Madin–Darby canine kidney cells treated with varying micromolar concentrations of compound 10 (ETCAR), compound 11 (ProTCAR), compound 12 (BrCAR) or ribavirin for 24 h

- UTP (Uridine triphosphate)
- ATP (Adenosine triphosphate)
- CTP (Cytidine triphosphate)
- GTP (Guanosine triphosphate)

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<th>Untreated control, %</th>
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27. Risel D, Strickler MD, Goldstein BM. Crystal structure of human inosine monophosphate dehydrogenase type II complexed with the MPA/NAD analog C2-MAD. *RSCB Protein Data Bank* 2004; doi: 10.2210/pdb1f7/pdb.


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