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QUARTERLY

ATP-binding domain of NTPase/helicase as a target for hepatitis C antiviral therapy★

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To enhance the inhibitory potential of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) *vs* hepatitis C virus (HCV) NTPase/helicase, ribavirin-5′-triphosphate (ribavirin-TP) was synthesized and investigated. Ribavirin-TP was prepared with the use of modified Yoshikawa-Ludwig-Mishra-Broom procedure (cf. Mishra & Broom, 1991, *J. Chem. Soc., Chem. Commun*, 1276–1277) involving phosphorylation of unprotected nucleoside.

Kinetic analysis revealed enhanced inhibitory potential of ribavirin-TP (IC_{50}=40 μM) as compared to ribavirin (IC_{50} > 500 μM). Analysis of the inhibition type by means of graphical methods showed a competitive type of inhibition with respect to ATP. In view of the relatively low specificity towards nucleoside-5′-triphosphates (NTP) of the viral NTPase/helicases, it could not be ruled out that the investigated enzyme hydrolyzed the ribavirin-TP to less potent products. Investigations on non- hydrolysable analogs of ribavirin-TP or ribavirin-5′-diphosphate (ribavirin-DP) are currently under way.

Hepatitis C virus is a small enveloped RNA virus identified as the major causative agent of non-A, non-B hepatitis [1, 2]. The viral genome encodes a polyprotein of approximately 3010 amino acids that is processed into structural proteins (core protein and envelope

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Abbreviations: ATP-γ-S, adenosine-5′(3-thiotriphosphate); FSBA, 5′-*O*-(4-fluorosulphonylbenzoyl)adenosine; HCV, hepatitis C virus; NTPase, nucleoside triphosphatase; NTP, nucleoside-5′-triphosphate(s); TMP, trimethylphosphate.

glycoproteins E1 and E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [3–5]. Among the proteins, NS3 appears to be the most promising target for antiviral agents because of the multiple enzymatic activities (serine protease and (NTPase)/ helicase [3–7]) associated with this protein.

Helicases are capable of enzymatically unwinding duplex DNA or RNA structures by disrupting the hydrogen bonds that keep the two strands together [8, 9]. Sequence comparisons have been used to group helicases into three superfamilies [8]. The NS3-like proteins of the bovine viral diarrhea pestivirus, plum pox potyvirus, vaccinia virus and of HCV belong to superfamily II (SFII) [8, 9]. All these proteins consist of three nearly equal-sized structural domains (I–III) separated by deep clefts [10, 11].

The RNA unwinding reaction is accomplished by the hydrolysis of γ-phosphate of NTP. The NTPase domain of the HCV helicase is clearly recognizable due to the presence of conserved amino-acid sequences associated with NTP binding [10, 12]. These include Walker motif A (motif I), which binds the terminal phosphate groups of NTP, and Walker motif B (motif II) that chelates the Mg^{2+} of the Mg–NTP complex [13]. These motifs have been found in many NTPases, helicases and ATP transphosphorylases e.g. adenylate and tymidine kinases [10, 11, 13, 14]. Mutations of residues in these motifs eliminate the NTP hydrolytic activity of the enzymes [14, 15]. Hydrolysis of the NTP triggers conformational changes in the molecule, e.g. the rotation of the domain II [10, 11]. These conformational changes are transmitted towards the domain II by a “switch region” containing a conserved sequence also known as motif III in the helicases of SFII [10, 11]. The structural proximity of the Walker motifs A and B to the motif III implies that the conformation rearrangements are dependent on the energy supplied by NTP hydrolysis [10]. In concordance with this is the observation that, on using a non-hydrolysable ATP analog (ATP-γ-S), only a low level of unwinding of HCV dsRNA was detected [16, 17]. Another non-hydrolysable ATP analog FSBA that covalently binds to the ATP-binding site of HCV NTPase/helicase [12] eliminated the helicase unwinding activity [18]. On the basis of these results, it seems that, compounds that reduce the accessibility of the ATP-binding site for ATP may be an important class of helicase inhibitors and potential anti-HCV agents. It should be mentioned that, at present, no really effective drug or vaccine against HCV infections does exist. The recently applied combination of ribavirin (**1**) (cf. Fig. 2) and α-interferon gives only 20% of cures. In order to explain the possible role of ribavirin in such combination, we decided to synthesize and investigate a metabolite of ribavirin (its 5′-triphosphate) which may be regarded as an analog of ATP.

MATERIALS AND METHODS

General methods. UV spectra were recorded on a Cary 3 instrument. Analytical TLC and PLC made use of Merck cellulose F plates (0.1 mm, No. 5565 and 0.5 mm, No. 5718, respectively) and Merck analytical silica gel plates (0.2 mm, No. 5554). Preparative paper chromatography was performed on Whatman 3MM paper. Chromatograms were developed in the following solvent systems (by vol.): (A) n-PrOH/H_2O/NH_4OH (11:2:7), (B) $CHCl_3$/MeOH (7:3).

All evaporations were made under vacuum at 35°C. Phosphorus was determined with the use of micromethod of Chen *et al.* [19]. Ribavirin and alkaline phosphatase were products of Sigma No. R-9644 and P-5778 respectively. All reagents and solvents were of analytical grade.

Synthesis of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide 5′-triphosphate, ribavirin-TP (4). Ribavirin-TP was synthesized (cf. Fig. 2) with the use of modified

Yoshikawa-Ludwig-Mishra-Broom procedure [20–22]. To a solution of ribavirin (**1**) (13 mg, 0.05 mmol) in anhydrous $(MeO)_3PO$ (0.5 ml) cooled to 0°C, $POCl_3$ (80 μl, 0.85 mmol) was added. The mixture was stirred at 0°C for 3 h, until phosphorylation was complete, as shown by thin-layer chromatography of a hydrolyzed aliquot on silica-gel with solvent A as developer. To a 0.5 M solution of bis-tri-n-butylammonium pyrophosphate a solution of Bu_3N in anhydrous DMF (0.5 ml) was added. The mixture was stirred for 5 min. The reaction was quenched by addition of 1 ml of aqueous $Et_3NH^+HCO_3^-$ (pH 7.3) at 20°C with stirring for 15 min. After evaporation *in vacuo,* the residue was resolved in MeOH, separated by PLC chromatography on cellulose F and precipitated from $MeOH\text{-}(CH_3)_2CO$. The precipitate was subjected to preparative chromatography on cellulose preparative plates employing solvent A. Subsequent elution with H_2O and lyophilisation gave pure ribavirin-TP (Yield: 30%, UV: λ_{max} (H_2O), 208 nm ε 12049).

Ribavirin-TP was digested completely by alkaline phosphatase to its mother nucleoside. R_f on cellulose F (solvent A): ribavirin = 0.78, ribavirin-TP = 0.44.

HCV NTPase/helicase isolation and purification. HCV NTPase/helicase (HCV-polyprotein-(1175–1657)) was expressed in *Escherichia coli* and purified as previously described [7]. The purity of the obtained protein was around 65–70%. The purification procedure was completed by gel exclusion chromatography on Superdex 200 (Pharmacia). The final preparation contains homogeneous HCV NTPase/helicase as demonstrated by silver stained SDS/PAGE.

ATPase assay. A standard ATPase assay was performed for 30 min at 30°C. HCV NTPase/helicase (20 pmol) was incubated in a reaction mixture (final volume 25 μl) that contained 20 mM Tris/HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol, 0.01% Triton X-100, 0.1 mg/ml bovine serum albumin and 11.0 μM [γ-^{32}P]ATP (0.5 μCi). The reaction was terminated by the addition of 0.5 ml of activated charcoal (2 mg/ml). After centrifugation at 10000 × ***g*** for 10 min, 50 μl aliquots of the supernatant were counted. Kinetic parameters were determined by non-linear-regression analysis using ENZFITTER (BioSoft) and SIGMA PLOT (Jandel Corp.).

RESULTS AND DISCUSSION

The NTPase/helicase used here is a homogeneous preparation of the enzyme (Fig. 1A) which has been used in several studies to characterize the properties of the NTPase and helicase [7, 12]. In our previous studies we have observed that the kinetic parameters of these enzymes are dependent on the reaction conditions [23, 24]. Therefore, it was necessary to optimize the conditions for the enzyme action before determining the inhibitory potential of the tested compounds. The ATPase activity of the HCV NTPase/helicase was tested as a function of the concentration of the divalent ions Mg^{2+} and Mn^{2+}. As shown, the ATPase reaction is strictly dependent on the divalent ion concentration. The two metal cations reveal optimal concentrations for the maximum of activity; 1–3 mM for Mg^{2+} and 0.3–0.5 mM for Mn^{2+} (Fig. 1B). The ATPase activity was measurable after 3–5 min of incubation, was linear for 15–60 min and approached its plateau after 90 min (Fig. 1C). The determination of the apparent K_m for ATP yielded a value of 11 μM (Fig. 1D). The optimal conditions determined for the enzyme differed from those determined by other authors. In contrast to HCV NTPase/helicase described by Suzich *et al.* [6] the ATPase activity of our enzyme is significantly stimulated by divalent ions also in the absence of polynucleotides. Further, unlike the enzyme used by Preugschat *et al.* [25], the ATPase activity of our HCV NTPase/helicase was only modestly susceptible to activation by oligomeric

nucleic acids (approx. 4-fold stimulation; not shown). Our observations indicate that these discrepancies might have resulted from differences in purity of the enzyme preparations.

We have performed experiments to determine the inhibitory character of ribavirin, a compound known to react with the nucleotide-binding site of different classes of enzymes [26], towards the HCV NTPase/helicase. At an ATP concentration equal to the K_m the ATPase activity of NTPase/helicase was only marginally inhibited by ribavirin (up to a concentration of 500 μM). The reduction of ATP concentration in the reaction mixture led to higher inhibition of the enzyme by ribavirin. At an ATP concentration of 0.01 μM (1/1000 of K_m value), an IC_{50} of 600–750 μM was measured. This dependence on ATP concentration may suggest a competitive mechanism of the inhibition in regard to ATP. However, the interaction of ribavirin with HCV NTPase/helicase is more complex. At higher concentrations of the compound (> 750 μM) and of ATP (> K_m) an activation of the ATPase activity of the enzyme was observed (not shown). The responsible mechanism remains to be resolved, however, our preliminary binding studies suggest the existence of a further functionally active NTP-binding site with apparently lower affinity for the nucleoside(s). The occupation of this site may lead to modulation of the accessibility of the "high affinity" ATP-binding site for ATP or to modulation of the ATPase activity of the enzyme by an alternative mechanism. This view is supported by a report of Porter [27] who provided kinetic evidence for the existence of two functionally active nucleotide-binding sites per monomer of the HCV NTPase/helicase.

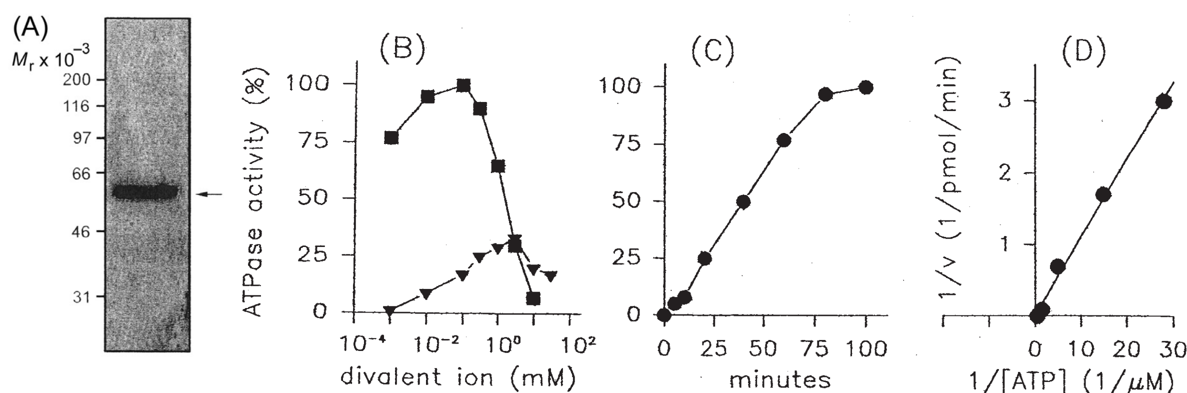

Figure 1. Analysis of the HCV NTPase/helicase used in this study.

(A) The purified enzyme preparation (10 pmol) was subjected to SDS/PAGE and stained with silver. Arrow indicates the position of NTPase/helicase. (B) Aliquots (20 pmol) of HCV NTPase/helicase were incubated in a reaction mixture containing ATP (11 μM) and increasing concentrations of Mg^{2+} (▼) or of Mn^{2+} (■) as indicated. (C) The reaction took place in the reaction mixture containing 11 μM ATP and 3 mM Mg^{2+} and was terminated at the times indicated. (D) The Lineweaver–Burk plot showing for the K_m of ATP. The reaction mixture contained the enzyme, 3 mM Mg^{2+} and varying concentrations of ATP.

Ribavirin (**1**) (Fig. 2) represents a compound that contains no phosphate groups and may be regarded as an analog of adenosine in which C(2)=N(3) fragment of adenine is removed and which appears to interact with the ATP-binding site of the HCV NTPase/helicase. In this context, it is conceivable that the introduction into the ribavirin molecule of 5′-diphosphate or 5′-triphosphate groups (containing β- and γ-phosphoric moieties) that interact with the Walker motifs of the

ATP-binding domain of the enzyme, could increase the affinity of the binding, and consequently, enhance the inhibitory potential of this compound. To test this hypothesis, ribavirin-TP (**4**) was synthesized and employed in inhibition studies.

sociated with ribavirin triphosphate, and to a lesser extent with 5′-mono- and diphosphates of ribavirin [29]. It has also been pointed out that, in healthy human volunteers, a fraction of the administered dose of ribavirin diffuses into red cells where it is phosphorylated, and re-

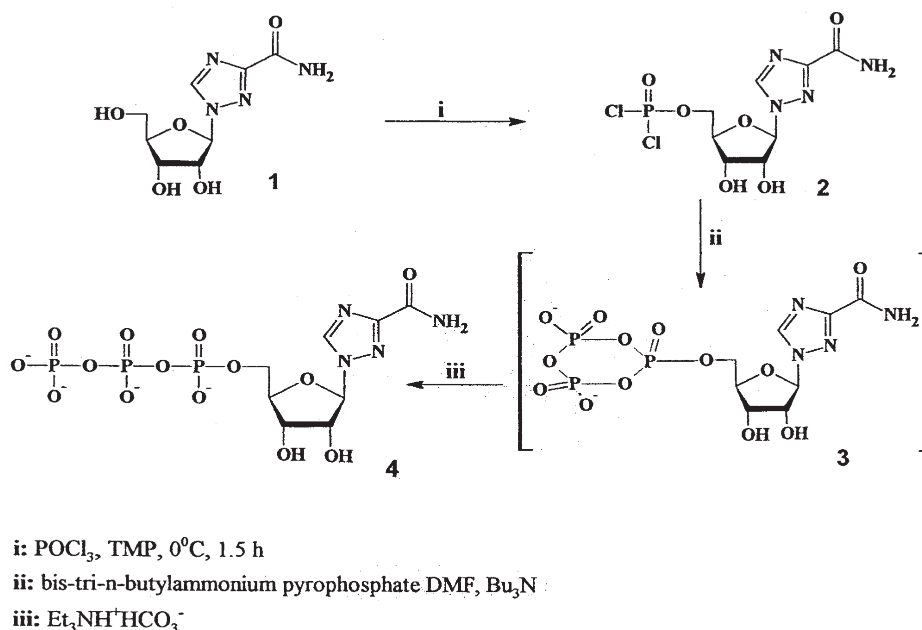

Figure 2. Synthesis of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide 5′-triphosphate (ribavirin-TP).

Ribavirin-TP (**4**) was prepared with the use of modified phosphorylation methods [20–22] (Fig. 2). One-pot phosphorylation procedure of unprotected nucleoside (**1**) employing excess of $POCl_3$ in TMP at 0°C, gave ribavirin phosphorodichloridate (**2**), which was converted to intermediate (**3**) by the reaction with bis-tri-n-tributylammonium pyrophosphate and excess of $POCl_3$ and DMF (Vilsmeier-Haack reagent). Then cleavage of (**3**) with aqueous triethylamine hydrogen carbonate gave ribavirin-TP (**4**) in 30% yield.

Phosphorylation of ribavirin *in vitro* and *in vivo* has been previously studied [28]. Miller *et al.* found [28] that tissues obtained from rats treated with ribavirin contained 5′-mono, di- and triphosphates of ribavirin plus additional metabolites. In further *in vivo* studies in mice with tritiated ribavirin it has been shown that intracellular radioactivity was principally as-

mains there until the red cells are removed by the normal mechanism. It was also shown that ribavirin-TP was by a wide margin the principal intracellular form of the drug, and the cellular concentration of ribavirin-TP equalled that of ATP [29]. One may suggest that this compound may be the form in which ribavirin exerts its anti-HCV activity.

The analysis of kinetic parameters of the inhibition of the NTPase/helicase by ribavirin-TP reveals (in comparison to ribavirin) an enhanced inhibitory potential of ribavirin-TP. At ATP concentrations equal to K_m, an IC_{50} value of 220 μM was measured. The inhibition of the enzyme by ribavirin-TP was more efficient at lower ATP concentrations (below 1/3–1/10 of the K_m measured for ATP). Thus, for example, at ATP concentrations of 0.01 μM the IC_{50} was 40 μM (Fig. 3A).

The kinetic parameters were subjected to graphical analysis as described by Dixon (reciprocal velocity *vs* inhibitor concentration) [30] (Fig. 3B) supplemented by replotting of the data as recommended by Cornish-Bowden (concentration of substrate/velocity *vs* inhibitor concentration) [31] (Fig. 3C). The evaluation of the plots indicated that the inhibition of HCV NTPase/helicase by ribavirin-TP is best approximated by a competitive mechanism in regard to ATP.

In this context it should be mentioned that, in contrast to ribavirin, high concentrations of ribavirin-TP enhanced only modestly the ATPase activity (not shown). In the light of this result it is tempting to suggest that the introduction of 5′-phosphate groups (containing β- and γ-phosphorus) into the ribavirin molecule reduces its affinity to the putative "low affinity" ATP-binding site.

In view of the relatively low specificity towards NTP of the viral NTPase/helicases [12, 25, 32–34], it can not be ruled out that the investigated enzyme catalyzes the hydrolysis of the ribavirin-TP to a derivative of poor potency. On the other hand, a synthesis of compounds that are more resistant to hydrolytic attack of these enzymes could result in obtaining potent inhibitors. Investigations in that directions are currently under way.

On the basis of the results described above, it seems that the blockade of the nucleoside-binding site of NTPase/helicase by nucleotide analogs with enhanced hydrolytic stability of the terminal phosphate group could be a convenient approach to inhibition of viral enzymes.

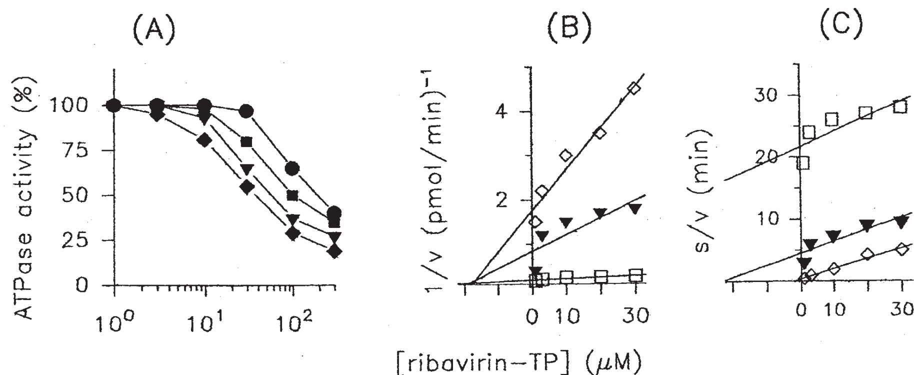

Figure 3. Inhibition of the ATPase reaction of HCV NTPase/helicase by ribavirin-TP and plots demonstrating the type of the inhibition relative to ATP.

(A) The ATPase reaction was performed at ATP concentrations corresponding to 1, 1/10, 1/100 and 1/1000 of K_m value, in the presence of increasing amounts of ribavirin-TP. The symbols used: ● (11 μM), ■ (1 μM), ▼ (100 nM) and ◆ (10 nM). The ATPase activity measured for each ATP concentration in the absence of the inhibitor was referred to as 100%. (B) The Dixon plots demonstrate the inhibitory effect of ribavirin-TP in the presence of ATP added at concentrations of 300 nM, 100 nM and 30 nM corresponding to 1/30 (•), 1/100 (▼) or 1/300 (•) of K_m value. (C) The respective supplementing double reciprocal plots according to Cornish-Bowden. The results shown are representative for three independent experiments.

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