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# Novel 1,2,4-triazole and imidazole derivatives of L-ascorbic and imino-ascorbic acid: Synthesis, anti-HCV and antitumor activity evaluations

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ABSTRACT

Several novel 1,2,4-triazole and imidazole L-ascorbic acid (**1**, **2**, **3**, **5**, **6** and **9**) and imino-ascorbic acid (**4**, **7** and **8**) derivatives were prepared and evaluated for their inhibitory activity against hepatitis C virus (HCV) replication and human tumour cell proliferation. Compounds **6** and **9** exerted the most pronounced cytostatic effects in all tumour cell lines tested, and were highly selective for human T-cell acute lymphoblastic leukaemia cells (CEM/0) with $IC_{50}$s of 10 ± 4 and 7.3 ± 0.1 μM, respectively. Unlike compound **9**, compound **6** showed no toxicity in human diploid fibroblasts. One of the possible mechanisms of action of compound **6** accounting for observed cytostatic activity towards haematological malignancies might be inhibition of inosine monophosphate dehydrogenase (IMPDH) activity, a key enzyme of de novo purine nucleotide biosynthesis providing the cells with precursors for DNA and RNA synthesis indispensable for cell growth and division, which has emerged as an important target for antileukemic therapy. In addition, this compound proved to be the most potent inhibitor of the hepatitis C virus replication as well. However, observed antiviral effect was most likely associated with the effect that the compound exerted on the host cell rather than with selective effect on the replication of the virus itself. In conclusion, results of this study put forward compound **6** as a potential novel antitumor agent (IMPDH inhibitor) for treating leukaemia. Its significant biological activity and low toxicity in human diploid fibroblasts encourage further development of this compound as a lead.



## 1. Introduction

Ascorbic acid is an essential micronutrient and is considered to have an antioxidant function in living systems. For the past several decades, ascorbic acid has risen to prominence as anticancer agent, although the mechanism underlying this biological effect still awaits to be unveiled. Recent studies showed that pharmacological concentrations of ascorbic acid selectively killed cancer but not normal cells by inducing programmed cell death (apoptosis) and pyknosis/necrosis, and that ascorbate-mediated cell death was due to protein-dependent extracellular $H_2O_2$ generation, via ascorbate radical formation from ascorbate as the electron donor.[1] Further in vivo studies provided evidence that single pharmacological dose of ascorbate produced sustained ascorbate radical and $H_2O_2$ formation selectively within interstitial fluids of tumours of mice bearing glioblastoma xenografts, and significantly decreased growth rates of ovarian, pancreatic, and glioblastoma tumours established in mice.[2] Additional confirmation of antitumor potential of ascorbic acid came from the study demonstrating that pharmacological doses of ascorbic acid suppress tumour growth and metastases in hormone-refractory prostate cancer.[3] In addition to anticancer properties, vitamin C and its derivatives have been ascribed antiviral activity as well.[4–6] In combination with several other bioactive compounds, ascorbic acid was shown to contribute to notable improvement of oxidative stress and immunological parameters in patients with chronic hepatitis C.[7] Standard therapy for treating hepatitis C is based on the combination of pegylated interferon alpha (pIFN-α) with ribavirin[8] (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide), a nucleoside analogue whose one of the many mechanisms of action also includes inhibition of inosine monophosphate dehydrogenase (IMPDH), a key enzyme of de novo purine nucleotide biosynthesis. Though standard therapy is effective in eradicating the hepatitis C virus in more than half of patients,[9] there is currently little prospect of an effective

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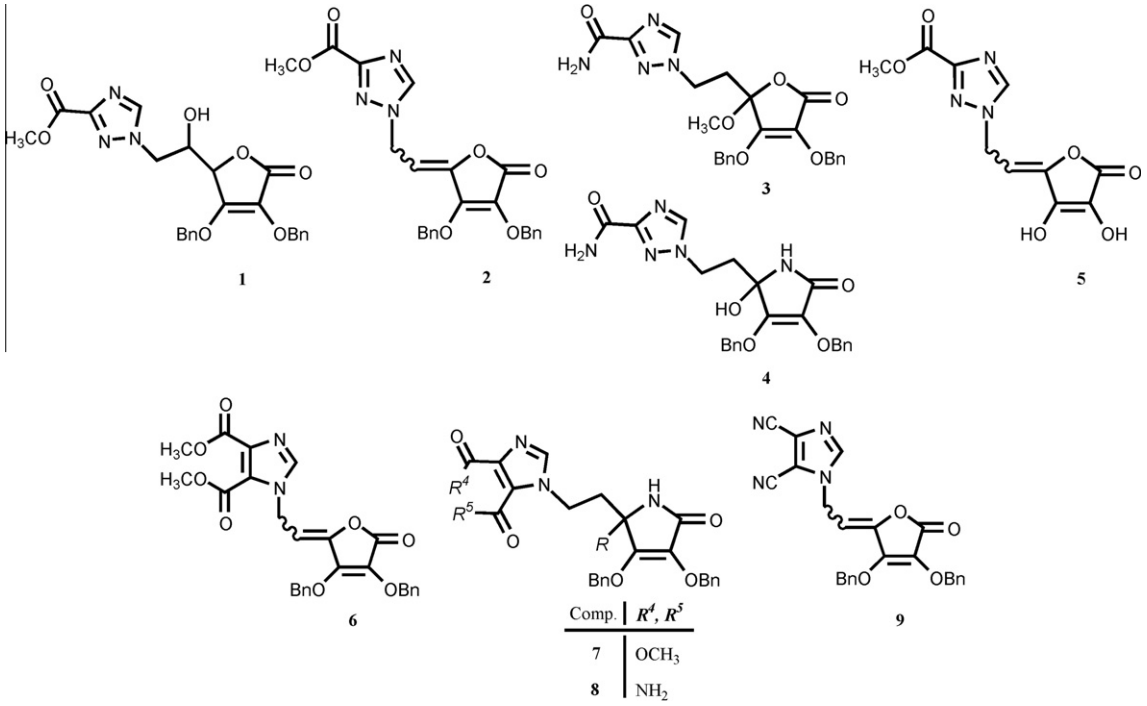



**Figure 1.** The novel 1,2,4-triazole-3-carboxamide and 4,5-disubstituted-imidazole L-ascorbic acid (**1**, **2**, **3**, **5**, **6** and **9**) and imino-ascorbic acid (**4**, **7** and **8**) derivatives.

vaccine for hepatitis C virus, which urges the need for developing more efficient antiviral drugs.[10]

In spite of long-standing research on ascorbic acid, its clinical utility has been limited by relatively low potency and in vivo efficacy therefore necessitating the need for development of novel derivatives with improved biological activity. Additionally, 1,2,4-triazole represents a unique template that is associated with antiviral, antibacterial, antifungal, anti-inflammatory and central nervous system (CNS) activity. Compounds incorporating a 1,2,4-triazol ring have also been shown to be antitumor agents.[11] Imidazole is an entity incorporated into many important biological molecules with a wide range of pharmacological activity. In the drug discovery the imidazole is the most important drug strategy and imidazoles are generally well known as anticancer agents.[12] Governed by this idea and in line with our previous study,[5] we efficiently synthesized a series of new molecules containing 1,2,4-triazole-3-carboxamide moiety (**1**–**5**), heterocyclic constituent of the ribavirin, or 4,5-disubstituted-imidazole ring (**6**–**9**) linked via ethylenic spacer to lactone or lactam moiety (Fig. 1). Newly prepared compounds were evaluated for their anti-HCV and anti-cancer properties, and their effect on IMPDH activity in mouse and human leukaemia cell lines was assessed.

## 2. Results and discussion

### 2.1. Chemistry

The novel 1,2,4-triazole and 4,5-disubstituted-imidazole L-ascorbic acid (**1**, **2**, **3**, **5**, **6** and **9**) and imino-ascorbic acid (**4**, **7** and **8**) derivatives are shown in Figure 1.

Compounds **1** and **2** were synthesized by standard Vorbrüggen condensation reaction conditions as depicted in Figure 2. Treatment of commercially available methyl 1*H*-1,2,4-triazol-3-carboxylate (**MT**) with excess hexamethyldisilazane (HMDS) and catalytic amount of ammonium sulphate resulted in the formation of the silylated derivative TMS-1,2,4-triazol-3-carboxylic methyl ester, which in subsequent reaction with 4-(5,6-epoxy)-2,3-di-*O*-benzyl-L-ascorbic acid (**EDAA**)[11] in dry acetonitrile, using trimethylsilyl trifluoromethanesulfonate (TMSOTf) as a catalyst, yielded compound **1** with a free hydroxy group at the *C*-5 of the ethylenic spacer. Treatment of silylated methyl 1*H*-1,2,4-triazole-3-carboxylate with 2,3-di-*O*-benzyl-5,6-di-*O*-acetyl-L-ascorbic acid (**dAdBA**)[13] in the same manner produced compound **2** as a mixture of (*Z*) and (*E*) isomers in 2:1 ratio.

Reaction of 1-[2-(3,4-bis-benzyloxy-5-oxo-5*H*-furan-2-ylidene)-ethyl]-1*H*-1,2,4-triazole-3-carboxylic acid methyl ester (**2**) with ammonia in anhydrous methanol gave compound **3** bearing a methoxy group at the *C*-4 of the lactone moiety, while the same reaction of **2** in dry dioxane yielded **4** with the hydroxyl group at *C*-4 of the lactam moiety (Fig. 2). We have found that in this reaction lactone ring is being rearranged to lactam ring. Such a rearrangement proceeds via nucleophilic addition of the ammonia to the carbonyl group with concomitant ring-opening of the lactone to the keto-enol-amide and subsequent intramolecular ring closure to give lactam as noted previously.[14] The structure of this compound and others obtained by reaction of ammonolysis was unambiguously confirmed by correlation 2D $^{1}H$–$^{15}N$ NMR spectroscopy indicating the presence of the imino group of the lactam ring.

Removal of benzyl protecting groups in compound **2** was achieved by using boron-trichloride to yield **5** bearing the free enolic hydroxyl groups at *C*-2 and *C*-3 of the lactone moiety as a mixture of (*Z*) and (*E*) isomers in 10:1 ratio.

Reaction of 4,5-dicarboxymethyl-(**DMI**) or 4,5-dicyano imidazole (**DCI**) with 5,6-di-*O*-acetyl-2,3-di-*O*-benzyl-L-ascorbic acid (**dAdBA**) and 1,8-diazobicyclo[5.4.0]undec-7-ene (**DBU**) afforded 4,5-dicarboxymethyl-(**6**) (*Z*/*E* = 10:1) and 4,5-dicyano-1,3-imidazole derivatives of L-ascorbic acid (**9**) (*Z*/*E* = 3:1) containing a double bond at the *C*4–*C*5 conjugated with the enone system of the lactone moiety. Introduction of gaseous ammonia in the solution of **6** in methanol and dioxane resulted in the addition of ammonia at the carbonyl group of the lactone moiety with concomitant ring opening and closure to lactam with free hydroxyl group at *C*-4 (**7**). Compound **8**, with the amido groups at the *C*-4 and *C*-5 of imidazole ring and hydroxyl

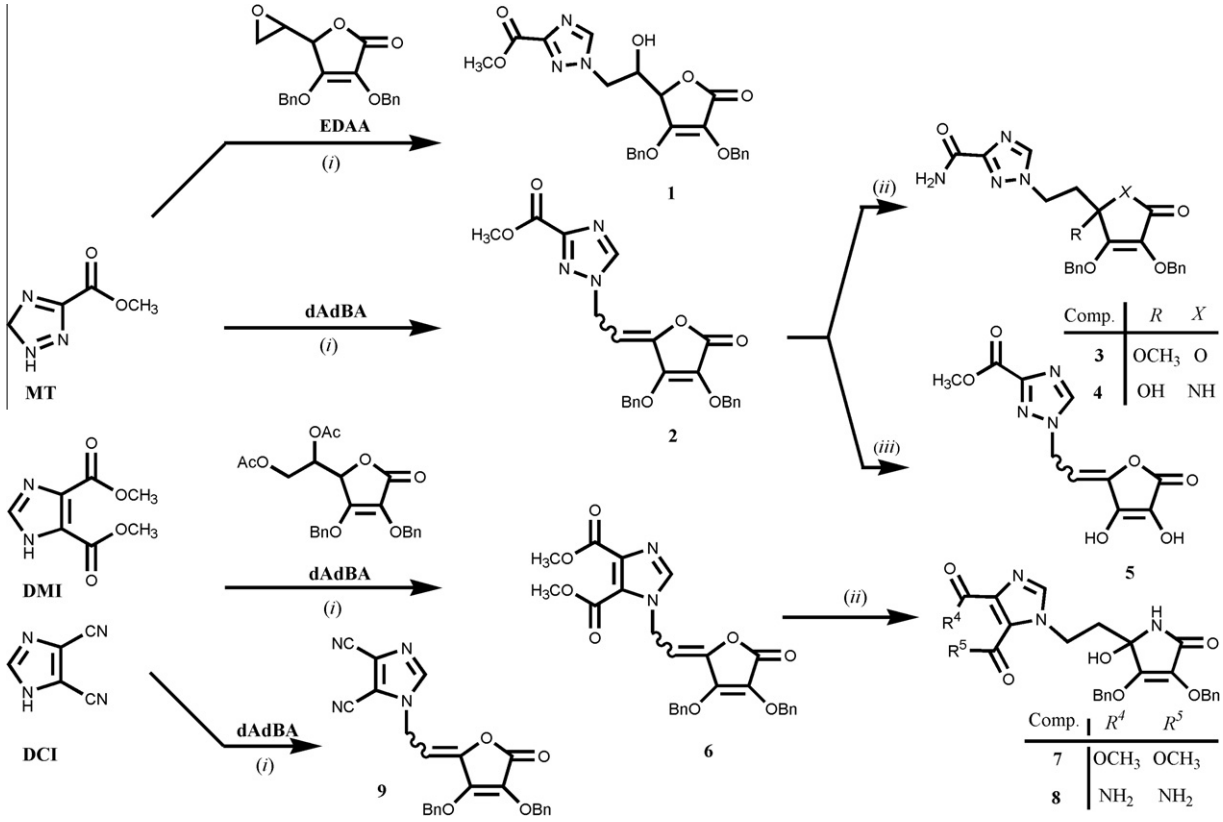



**Figure 2.** Synthesis of 1,2,4-triazole and 4,5-disubstituted-imidazole L-ascorbic acid (**1**, **2**, **3**, **5**, **6** and **9**) and imino-ascorbic acid (**4**, **7** and **8**) derivatives. Reagents and conditions: (i) HMDS/$(NH_4)_2SO_4$/argon/reflux 12 h; TMS-triflate/dry acetonitrile/55-70 °C/12 h; (ii) $NH_3$/MeOH-dioxane/rt/24 h; (iii) $BCl_3/CH_2Cl_2/-78$ °C/2 h.

group at the *C*-4 of the lactam moiety, broke forth from prolonged reaction time (96 h) at room temperature.

It is interesting to note that removal of *O*-benzyl protective groups in triazole (**4**) and imidazole (**7** and **8**) 3-pyrroline-2-on derivatives containing a free hydroxyl group at *C*-4 of the lactam moiety by using either Lewis acid ($BCl_3$)-catalyzed hydrolysis, catalytic hydrogenation with $Pd/H_2$ or microwave-assisted hydrogenation with $Pd_{ENC}$ didn't yield the desired derivatives with free enolic hydroxyl groups which may be due to instability of the lactam moiety caused by the presence of proton on nitrogen and the hydroxyl group at the position *C*-4.

## 2.2. Structural and conformational properties

The chemical identities of compounds **1**–**9** were confirmed by $^1H$, $^{13}C$ and $^{15}N$ NMR measurements. Proton and carbon NMR chemical shifts are reported in the Experimental section. Noteworthy, the correlation signals observed in $^1H$–$^{15}N$ HSQC and HMBC spectra confirmed the presence of the lactam functionality in **8**. NH proton at $\delta_H$ 8.15 ppm showed cross-peak at $\delta_N$ 127.0 ppm (Fig. S1). The exchangeable proton at $\delta_H$ 6.13 ppm showed no correlation signal in $^{15}N$ HSQC and was assigned to the *C*-4′-hydroxyl group. The remaining cross-peaks observed in $^{15}N$ HSQC spectrum were attributed to the two amide groups in the imidazole ring ($\delta_N$ 109.4 and 114.0 ppm, Fig. S1). Additionally, the presence of the lactam ring in **8** was supported by more shielded C4′ atom ($\delta_C$ 81.85 ppm) in comparison with the chemical shift of C4′ atom in lactone derivative **3** ($\delta_C$ 102.24 ppm). In a similar manner, the lactam functionalities were confirmed by 2D $^{15}N$ NMR experiments in compounds **4** and **7** (Figs. S2 and S3, respectively).

Conformational properties of compound **2** were assessed using long range $J_{CH}$ coupling constants, which were established by J-HMBC experiment. The $^3J_{C3'H5'}$ coupling constant of 2.4 Hz corresponded to (*Z*) geometry along the C4′=C5′ double bond, while larger coupling constant of 6.2 Hz indicated (*E*) geometry (Fig. 3a). Weak NOE enhancements between H-5 and H-5′ suggested predominance of an orientation, where H-5 and H-5′ protons are spatially close in both isomers (Fig. 3a). Likewise, *Z* and *E* isomers for compound **9** were distinguished with the use of long-range proton–carbon coupling constants. $^3J_{C3'H5'}$ of 3.0 Hz is in agreement with (*Z*) configuration, whereas coupling constant of 6.5 Hz suggested (*E*) geometry along the C4′=C5′ double bond.

Major chemical shift differences of geminal 5-$CONH_2$ protons ($\Delta\delta_H$ = 3 ppm) were observed in **8**. These observations suggested involvement of more deshielded proton in hydrogen bond leading to stabilization of conformer presented in Figure 3b. Such conformation is supported by NOE enhancements (Fig. 3b).

## 2.3. Biological results

### 2.3.1. Anti-HCV activity

The novel 1,2,4-triazole and 4,5-disubstituted-imidazole L-ascorbic acid (**1**, **2**, **3**, **5**, **6** and **9**) and imino-ascorbic acid (**4**, **7** and **8**) derivatives were evaluated for their inhibition of the hepatitis C virus replication in the Huh 5-2 replicon system. The 4,5-dicarboxymethyl-imidazole L-ascorbic acid derivative (**6**) proved to be the most potent compound (Table 1) with $EC_{50}$ of 13.3 μg/mL as deduced from the dose–response curve. Based upon the measurement of its antimetabolic effect ($CC_{50}$ of 70.5 μg/mL), a selectivity index of 5.3 could be calculated. However, bearing in mind poor antimetabolic effect of this compound at concentrations that induce observed antiviral effect, the latter might be ascribed to non-specific pleiotropic effects that the compound exerts on the host cells rather than to selective effect on the multiplication

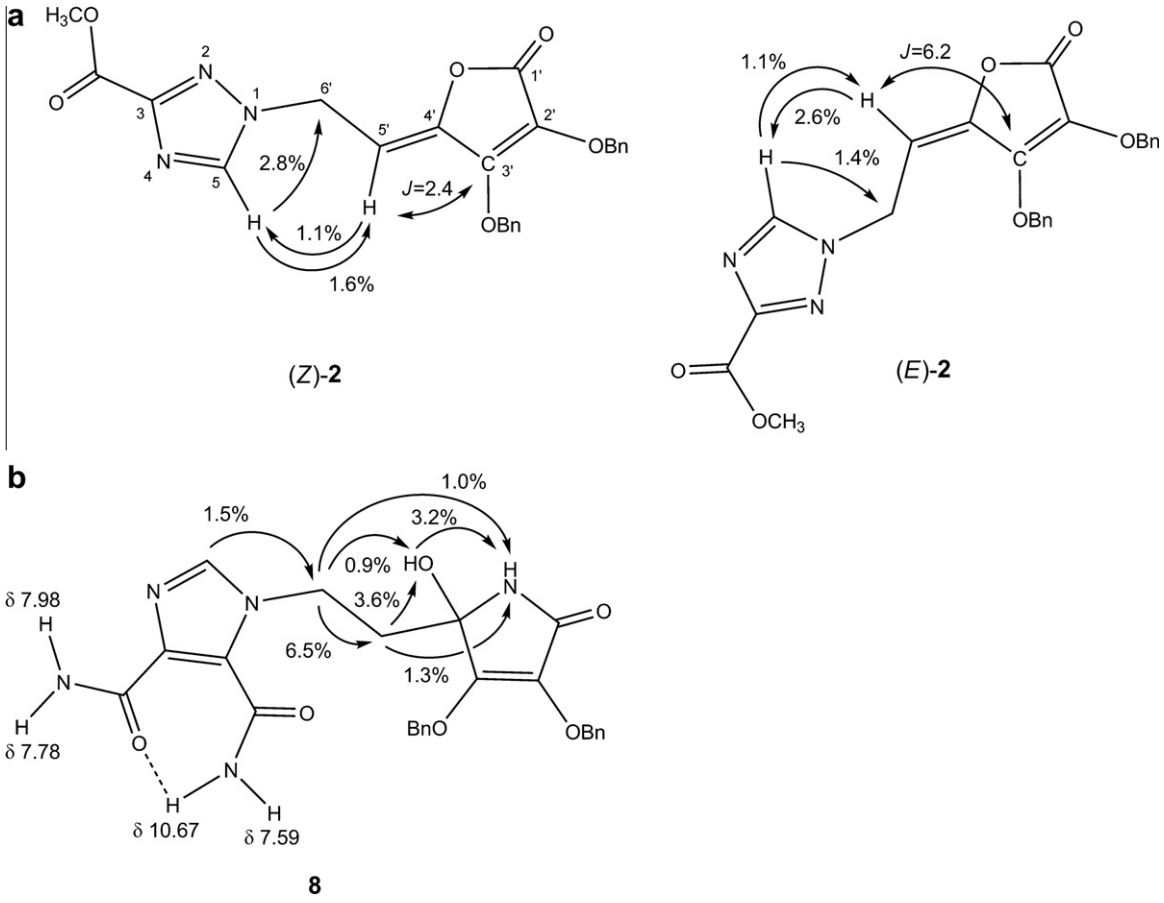



**Figure 3.** (a) (*Z*) and (*E*) isomers of **2** with the corresponding $^{3}J_{CH}$ coupling constants and key NOE enhancements. (b) Predominant conformation of **8** established by NOE enhancements and chemical shifts of amino protons suggesting stabilization through O...H–N hydrogen bonds.

of the replicon itself. Therefore, it seems implausible to perceive this compound as a selective inhibitor of the hepatitis C virus replication in the Huh 5-2 replicon system.

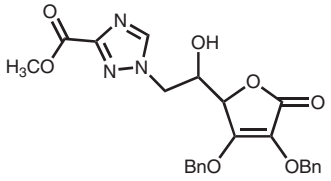
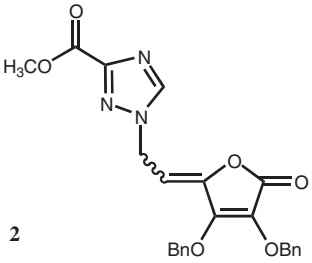
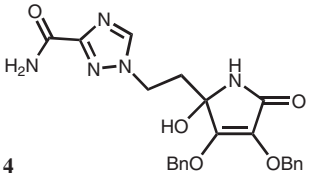
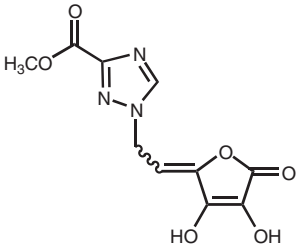
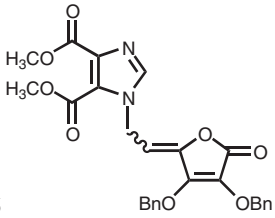
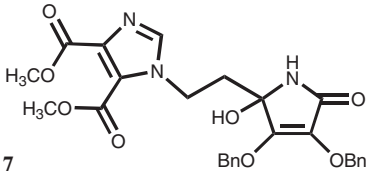
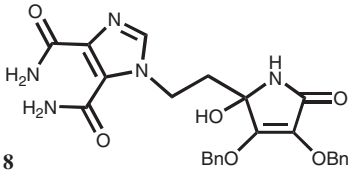
### 2.3.2. Cytostatic activity

The synthesized compounds **1**–**2** and **4**–**9** were evaluated in vitro for their cytostatic effect against murine leukaemia (L1210) and human tumour cell lines including human T-cell acute lymphoblastic leukaemia (CEM), cervical carcinoma (HeLa), metastatic breast epithelial adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), colorectal adenocarcinoma (SW620) and pancreatic carcinoma (MiaPaCa-2) along with human normal diploid fibroblasts (WI38) (Table 2). Compounds **6** and **9** exerted the most pronounced cytostatic effects in all tumour cell lines tested, and were highly selective for human T-cell acute lymphoblastic leukaemia cells (CEM/0) with $IC_{50}$s of 10 ± 4 and 7.3 ± 0.1 μM, respectively. However, unlike compound **9**, compound **6** was not cytotoxic to human fibroblasts. In addition, compound **4** containing 3-carboxyamide-1,2,4-triazole ring connected via ethylenic spacer to lactam moiety bearing *C*-4 substituted free hydroxyl group showed selective but rather weak antiproliferative activity in MCF-7 and HepG2 cells with $IC_{50}$s of 61 ± 0.2 and 66 ± 0.6 μM, respectively, and no cytotoxicity in WI38.

### 2.3.3. IMPDH activity assays

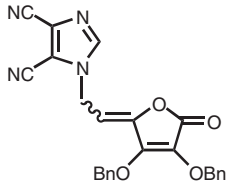
Inosine 5′-monophosphate dehydrogenase (IMPDH, E.C.1.1.1.205), a NAD dependent enzyme that controls de novo synthesis of purine nucleotides, catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP), which is then converted to guanosine 5′-monophosphate (GMP) by GMP synthase. IMP also serves as a substrate for the biosynthesis of adenosine 5′-monophosphate (AMP). Due to its both, antiproliferative and differentiation-inducing effects, IMPDH inhibitors represent a promising class of antileukemic agents.[15] In order to determine whether newly prepared compounds exert any inhibitory effect on IMPDH activity in L1210 and CEM leukaemia cell lines, we measured tritium release from [2,8-$^{3}$H]hypoxanthine in the absence (control) or presence of different concentrations of these compounds. Ribavirin, a well-known IMPDH inhibitor, was included as a reference drug. As expected, this compound markedly inhibited IMPDH activity in a dose-dependent manner in both tumour cell lines (Fig. 4). Its $IC_{50}$ was approximately 1–2.5 μM, regardless of measurement time points (20, 40 or 60 min). Among the tested ascorbic acid derivatives, compounds **2**, and **8** as well as compound **6** at a lower extent, showed inhibitory potential in both leukaemia cell lines, albeit at concentrations that were approximately 50- to 100-fold higher than those of ribavirin effective concentrations ($IC_{50}$: 100–250 μM) (Fig. 4), pointing to their ability to interfere with purine biosynthesis. It should be noted that IMPDH exists in two isoforms, namely type I ('housekeeping') that is constitutively expressed in normal and neoplastic cells, and type II whose expression is up-regulated in human neoplastic cell lines and in leukaemic cells from patients with chronic granulocytic, lymphocytic and acute myeloid leukaemias.[15] Moreover, inhibitors of IMPDH can alternatively suppress the enzyme activity by binding at the cofactor (NAD) site as well. For example, ribavirin is converted in vivo into its 5′-monophosphate, which mimics IMP and binds at the substrate site. Although our data clearly speak in

**Table 1**
Antiviral effect of compounds **1**–**2** and **4**–**9** on hepatitis C virus replication in the Huh 5-2 replicon system

| Compd | Antimetabolic and antiviral effect | | | |
|---|---|---|---|---|
| | $CC_{50}$[a] | $EC_{50}$[b] | $EC_{90}$[c] | Unit |
|  **1** | >100 | 62.1 | ND[d] | μg/mL |
|  **2** | >100 | 78.5 | >100 | μg/mL |
|  **4** | >100 | >100 | >100 | μg/mL |
|  **5** | >100 | 61.3 | ND | μg/mL |
|  **6** | 70.5 | 13.3 | 22.7 | μg/mL |
|  **7** | >100 | 36.6 | 81.3 | μg/mL |
|  **8** | >100 | >100 | >100 | μg/mL |

(*continued on next page*)

**Table 1** (*continued*)

| Compd | Antimetabolic and antiviral effect | | | |
|---|---|---|---|---|
| | $CC_{50}$[a] | $EC_{50}$[b] | $EC_{90}$[c] | Unit |
|  **9** | >100 | >100 | >100 | μg/mL |

[a] $CC_{50}$ = 50% cytostatic/cytotoxic concentration (concentration at which 50% adverse effect is observed on the host cell).
[b] $EC_{50}$ = 50% effective concentration (concentration at which 50% inhibition of virus replication is observed).
[c] $EC_{90}$ = 90% effective concentration (concentration at which 90% inhibition of virus replication is observed).
[d] ND = not determined.

**Table 2**
Inhibitory effects of compounds **1**–**2** and **4**–**9** on the growth of malignant tumour cell lines in comparison with their effect on normal diploid human fibroblasts (WI38)

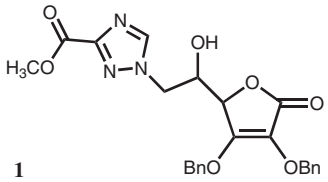
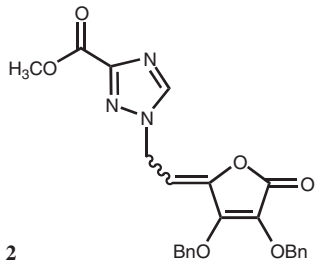
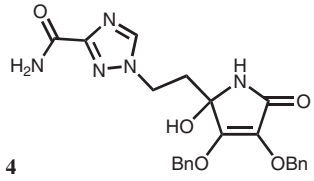
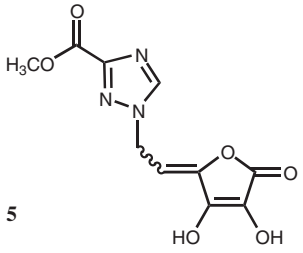
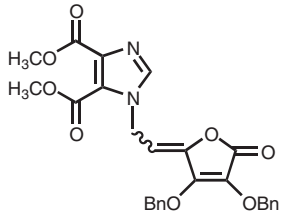
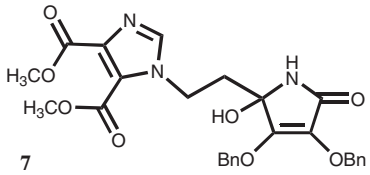
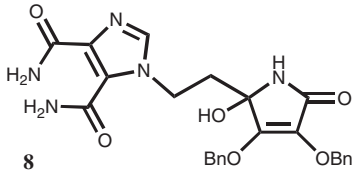
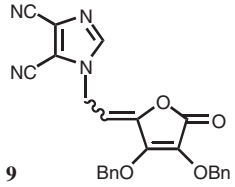
| Compd | Tumour cell growth $IC_{50}$[a] (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | L1210/0 | CEM/0 | HeLa | MCF-7 | HepG2 | SW620 | MiaPaCa-2 | WI38 |
|  **1** | >100 | >100 | >100 | >100 | >100 | >100 | >100 | >100 |
|  **2** | 103 ± 0.3 | 44 ± 0.4 | 97 ± 0.4 | 54 ± 0.4 | 70 ± 0.7 | >100 | 80 ± 0.5 | >100 |
|  **4** | ⩾500 | 445 ± 77 | >100 | 61 ± 0.2 | 66 ± 0.6 | >100 | >100 | >100 |
|  **5** | 370 ± 183 | >500 | >100 | >100 | >100 | >100 | >100 | >100 |
|  **6** | 32 ± 11 | 10 ± 4 | 34 ± 0.9 | 52 ± 0.8 | 55 ± 1.3 | 49 ± 0.9 | 33 ± 1.1 | >100 |

**Table 2** (*continued*)

| Compd | Tumour cell growth $IC_{50}$[a] (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | L1210/0 | CEM/0 | HeLa | MCF-7 | HepG2 | SW620 | MiaPaCa-2 | WI38 |
|  **7** | 46 ± 6 | 35 ± 1 | 81 ± 0.4 | >100 | >100 | >100 | 47 ± 0.6 | 52 ± 0.4 |
|  **8** | 198 ± 1 | 154 ± 9 | >100 | >100 | >100 | >100 | >100 | 98 ± 0.3 |
|  **9** | 21 ± 6 | 7.3 ± 0.1 | 59 ± 0.6 | 63 ± 0.6 | 92 ± 0.6 | 73 ± 0.7 | 36 ± 1.0 | 73 ± 0.5 |
| Doxorubicin | 1.3 ± 0.1 | 0.39 ± 0.21 | 0.06 ± 0.01 | 0.06 ± 0.01 | 0.05 ± 0.002 | 0.04 ± 0.005 | 0.03 ± 0.004 | 0.04 ± 0.006 |
| Ribavirin (for L1210 and CEM) *5-Fluoruracil* (for other cell lines) | 22 ± 5 | 63 ± 14 | 14.93 ± 1.0 | 66.91 ± 1.16 | 56.2 ± 1.39 | 6.68 ± 0.12 | 11.67 ± 0.53 | 24.66 ± 3.37 |

[a] $IC_{50}$; 50% inhibitory concentration, or compound concentration required to inhibit tumour cell proliferation by 50%.

favour of compound **6** as a specific IMPDH inhibitor in human leukaemia cells, the precise binding mode of this compound to IMPDH remains speculative until additional crystallographic studies will be performed.

## 3. Conclusion

We have synthesized new 1*H*-1,2,4-triazole and imidazole L-ascorbic acid (**1**, **2**, **3**, **5**, **6** and **9**) and imino-ascorbic acid (**4**, **7** and **8**) derivatives and assed their antiviral and anticancer properties. Among those, compounds **9** and **6** were the most potent antitumor agents with high selectivity towards human T-cell acute lymphoblastic leukaemia cells (CEM). However, compound **6** is preferred over compound **9** due to its non-toxic effects on human diploid fibroblasts. Its growth inhibitory activity might be partially ascribed to inhibition of IMPDH activity, a key enzyme in providing an adequate pool of purine nucleotides for cell proliferation, cell signalling, and as an energy source. Consequently, inhibition of IMPDH causes a variety of biological responses, and this enzyme has emerged as a major target for antiviral, antileukemic and immunosuppressive therapies. One of the most potent inhibitors of tumour cell proliferation, namely compound **6**, ranked also among the most potent inhibitors of the hepatitis C virus replication, and was comparable in its cytostatic potency to ribavirin, a standard agent in treating chronic hepatitis C virus infection. However, observed antiviral effect is most likely associated with the effect the compound exerts on the host cell rather than with selective effect on the replication of the virus itself. In conclusion, several interesting properties including highly specific growth inhibitory activity towards tumour cells, in particular towards haematological malignancies and low cytotoxicity make compound **6** attractive drug candidate to be further investigated from therapeutic viewpoint. Next challenge would be to synthesize 5-ethynylimidazole derivatives of L-ascorbic acid to reveal whether they can covalently modify the active cysteine site on IMPDH as another IMPDH inhibitor, EICAR does in the IMP binding pocket.

## 4. Experimental section

### 4.1. Materials and general methods

All commercially available chemicals were purchased from Sigma Aldrich (Germany) and used without purification. All solvents were analytical grade purity and dried. Acetonitrile ($CH_3CN$) was refluxed over calcium hydride ($CaH_2$), distilled and stored over 3 Å molecular sieves. Methanol ($CH_3OH$) was stored over 3 Å molecular sieves. Dioxane was dried with sodium and stored over 4 Å molecular sieves. Dichloromethane ($CH_2Cl_2$) was refluxed over phosphorus pentoxide ($P_2O_5$), distilled and stored over 4 Å molecular sieves.

Merck silica gel 60 $F_{254}$ plates were used for thin-layer chromatography. Column chromatography was performed with Merck silica gel (0.063–0.200 mm), with petroleum ether/ethylacetate (1:1) and dichloromethane/methanol (70:1, 50:1, 20:1) as eluents.

Melting points were determined on a Kofler micro hot-stage instrument (Reichter, Wien) and were uncorrected. $^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Gemini 300 spectrometer (Institute Ruđer Bošković, Zagreb) and Varian NMR System 600 and Varian Unity Inova 300 and Agilent Technologies DD2 300 MHz NMR spectrometers (National Institute of Chemistry, Ljubljana, Slovenia) Samples were measured in $CDCl_3$ and DMSO-$d_6$ solutions at 25 °C in 5 mm NMR tubes. Chemical shifts ($\delta$) in ppm were referred to TMS. High performance LC was performed on Agilent 1100 series system with UV detection (photodiode array detector) using Zorbax C18 reverse-phase analytical column (2.1 × 30 mm; 3.5 μm). All compound used for biological evaluation showed >95% purity in this HPLC system. The electron impact mass spectra and the purity of compounds were assessed by using Agilent

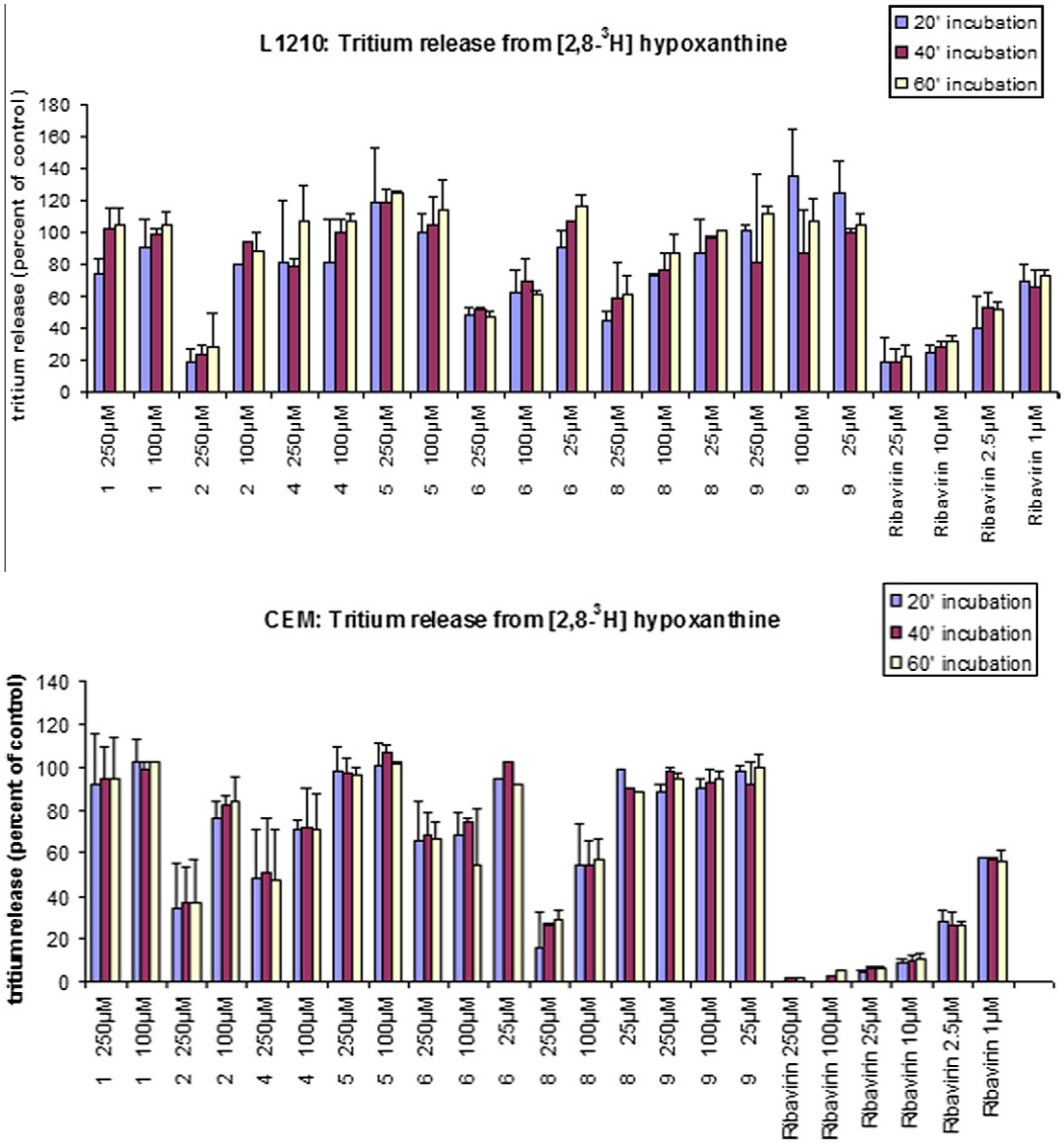



**Figure 4.** IMP dehydrogenase activity measurements in L1210 and CEM cell cultures in the presence of ribavirin and the test compounds.

Technologies 6410 Triple Quad LC/MS instrument equipped with electrospray interface and triple quadrupole analyzer (LC–MS/MS).

## 4.2. Synthesis

### 4.2.1. 1-[2-(3,4-Bis-benzyloxy-5-oxo-2,5-dihydro-furan-2-yl)-2-hydroxy-ethyl]-1*H*-1,2,4-triazole-3-carboxylic acid methyl ester (1)

1*H*-1,2,4-triazol-3-carboxylate (643.25 mg; 5.06 mmol), $(NH_4)_2SO_4$ (38.64 mg; 0.34 mmol) and hexamethyldisilazane (HMDS; 18.20 mL) were heated under reflux in argon atmosphere overnight. Excess HMDS was evaporated under reduced pressure. Silylated 1*H*-1,2,4-triazol-3-carboxylate, 4-(5,6-epoxy)-2,3-di-*O*,*O*-benzyl-L-ascorbic acid (427.67 mg; 1.26 mmol) and TMSOTf (0.64 mL; 4 × 0.16 mL) were heated at 55 °C in anhydrous acetonitrile (6.40 mL) under argon atmosphere. The progress of the reaction was monitored by TLC [$CH_2Cl_2$/MeOH = 50:1] and after stirring overnight, reaction mixture was concentrated in vacuum and the resulting residue was extracted with dichloromethane and saturated solution of $NaHCO_3$. Organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. Purification on a silica gel column chromatography using dichloromethane/methanol = 70:1, gave **1** as a white oil (22.5 mg; 3.95%); MS *m*/*z* 450.5 [$M^+$].

$^1$H NMR (DMSO-$d_6$): $\delta$ 3.88 ($OCH_3$, 3H, s), 4.08 (H5′, 1H, m), 4.36 (H6′α, 2 × 1H, 2 × d, *J* = 13.8, 10.0), 5.01 (H4′, 1H, d, *J* = 1.0), 4.59 (H6′β, 2 × 1H, 2 × d, *J* = 13.8, 2.6), 4.95/4.97 (2′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.2/11.2), 5.18/5.27 (3′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.7/11.7), 5.62 (5′-OH, 1H, d, *J* = 6.9), 7.29–7.41 (Ph, 10H, m), 8.74 ppm (H5, 1H, s).

$^{13}$C NMR (DMSO): $\delta$ 49.15 (C6′), 53.10 ($OCH_3$), 67.27 (C5′), 73.27 (2′-$OCH_2$), 74.11 (3′-$OCH_2$), 76.22 (C4′), 121.46 (C2′), 128.26–129.15 ($C_6H_5$), 136.06 ($C_6H_5$), 136.60 ($C_6H_5$), 141.36 (C3′), 144.83 (C3), 147.35 (C5), 157.70 (3-CO), 169.46 ppm (C-1′).

Elemental Anal. Calcd for $C_{24}H_{23}N_3O_7$: C 61.93, H 4.98, N 9.03, Found: C 62.05, H 4.99, N 9.05.

### 4.2.2. 1-[2-(3,4-Bis-benzyloxy-5-oxo-5*H*-furan-2-ylidene)-ethyl]-1*H*-1,2,4-triazole-3-carboxylic acid methyl ester (2)

Mixture of 1*H*-1,2,4-triazol-3-carboxylate (2.00 g; 15.74 mol), $(NH_4)_2SO_4$ (246 mg; 15.74 mol) and HMDS (50 mL) was heated under reflux for 3 h under argon atmosphere. Excess HMDS was

evaporated under reduced pressure and the resulting yellow oil of silylated 1*H*-1,2,4-triazol-3-carboxylate, 2,3-di-*O*,*O*-benzyl-5,6-di-*O*,*O*-acetyl-L-ascorbic acid (4.6 g; 10.44 mmol) and TMSOTf (4.00 mL; 2 × 2.00 mL) was heated at 55 °C in dry acetonitril (30 mL) under argon atmosphere overnight. Progress of the reaction was monitored by TLC [petroleum/ethylacetate = 1:1]. Reaction mixture was concentrated by evaporation and the crude product thus obtained extracted with dichloromethane and saturated $NaHCO_3$ solution. Organic layer was dried over $MgSO_4$ and concentrated in vacuo. Crude product was purified by silica gel column chromatography using petroleum ether/ethylacetate = 1:1 as eluent to give **2** as a mixture of (*Z*)- and (*E*)-isomers (2:1), as a dark-yellow oil (625.3 mg; 13.4%); MS *m*/*z* 447.8 [$M^+$].

$^1$H NMR ($CDCl_3$): (***Z***)-**2**: $\delta$ 4.00 ($OCH_3$, 3H, s), 5.18 (H6′, 2H, d, *J* = 7.6 Hz), 5.21 (2′-$OCH_2$, 2H, s), 5.23 (3′-$OCH_2$, 2H, s), 5.49 (H5′, 1H, t, *J* = 7.6 Hz), 7.2–7.4 (Ph, 10H+10H (***E***)-**2**, m), 8.31 (H5, 1H, s). (***E***)-**2**: $\delta$ 3.97 ($OCH_3$, 3H, s), 5.21 (H6′, 2H, d, *J* = 8.4 Hz), 5.30 (2′-$OCH_2$ and 3′-$OCH_2$, 4H, s), 5.76 (H5′, 1H, t, *J* = 8.4 Hz), 7.2–7.4 (Ph, 10H+10H (***Z***)-**2**, m), 7.87 (H5, 1H, s).

$^{13}$C NMR ($CDCl_3$): (***Z***)-**2**: $\delta$ 40.96 (C6′), 53.24 ($OCH_3$), 73.69 (3′-$OCH_2$), 74.25 (2′-$OCH_2$), 100.31 (C5′), 123.99 (C2′), 127.8–129.8 ($C_6H_5$), 135.21 ($C_6H_5$), 135.61 ($C_6H_5$), 144.29 (C3), 145.40 (C4′), 146.37 (C5), 147.62 (C3′), 158.45 (3-CO), 163.59 (C1′). (***E***)-**2**: $\delta$ 41.27 (C6′), 53.18 ($OCH_3$), 74.23/74.67 (2′-$OCH_2$/3′-$OCH_2$), 105.46 (C5′), 125.90 (C2′), 127.8–129.8 ($C_6H_5$), 134.92 ($C_6H_5$), 135.54 ($C_6H_5$), 144.21 (C3), 145.11 (C4′), 145.85 (C5), 147.83 (C3′), 158.35 (3-CO), 163.54 (C1′).

Elemental Anal. Calcd For $C_{24}H_{21}N_3O_6$: C 64.42, H 4.73, N 9.39, Found: C 64.27, H 4.74, N 9.37.

#### 4.2.3. 1-[2-(3,4-Bis-benzyloxy-2-methoxy-5-oxo-2,5-dihydro-furan-2-yl)-ethyl]-1*H*-[1,2,4]triazole-3-carboxylic acid amide (3)

Ammonia was introduced in a stirred solution of compound **2** (145 mg; 0.324 mmol) in anhydrous methanol (10 mL) at 0 °C. After saturation the reaction mixture was stirred overnight at room temperature. Methanol and ammonia were removed in vacuum. Purification of crude product by silica gel column chromatography using dichloromethane/methanol = 50:1 gave desired compound **3** as a yellow oil (46.6 mg; 31.1%); MS *m*/*z* 465.2 [M+1].

$^1$H NMR ($CDCl_3$): $\delta$ 2.18/2.42 (H5′, 2 × 1H, 2 × m), 2.96 (4′-$OCH_3$, 3H, s), 4.48/4.50 (H6′, 2 × 1H, 2 × m), 5.14/5.27 (2′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.4/11.4 Hz), 5.20 (3′-$OCH_2$, 2H, s), 7.2–7.4 (Ph, 10H, m), 7.83/8.21 (3-$CONH_2$) 8.10 ppm (H5, 1H, s).

$^{13}$C NMR ($CDCl_3$): $\delta$ 37.44 (C5′), 41.29 (C6′), 50.69 (4′-$OCH_3$), 73.82 (3′-$OCH_2$), 73.93 (2′-$OCH_2$), 102.24 (C4′), 121.92 (C2′), 128.1–129.6 ($C_6H_5$), 135.24 ($C_6H_5$), 135.74 ($C_6H_5$), 145.77 (C3), 147.26 (C5), 154.00 (C3′), 158.84 (3-CO), 166.26 ppm (C1′).

Elemental Anal. Calcd For $C_{24}H_{24}N_4O_6$: C 62.02, H 5.21, N 12.06, Found: C 62.19, H 5.21, N 12.09.

#### 4.2.4. 1-[2-(3,4-Bis-benzyloxy-2-hydroxy-5-oxo-2,5-dihydro-1*H*-pyrrol-2-yl)-ethyl]-1*H*-1,2,4-triazole-3-carboxylic acid amide (4)

Compound **2** (512.5 mg; 1.145 mmol) was dissolved in dry dioxane (25 mL) at 0 °C and ammonia was introduced until saturation. Reaction mixture was then stirred at room temperature overnight. Anhydrous methanol (25 mL) was added to a stirred solution at 0 °C and ammonia again introduced until saturation. After stirring overnight at room temperature, solvents and ammonia were removed under reduced pressure. The resulting residue was purified by silica gel column chromatography ($CH_2Cl_2$/MeOH = 50:1) to give **4** as a white crystals (193.1 mg; 37.4%, mp = 156–158 °C); MS *m*/*z* 449.9 [$M^+$].

$^1$H NMR (DMSO-$d_6$): $\delta$ 2.13 (H5′, 2H, m), 4.33 (H6′, 2H, m), 4.99/5.01 (2′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.2/11.2 Hz), 5.05/5.14 (3′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.9/11.9 Hz), 6.18 (OH, 1H, s), 7.3–7.4 (Ph, 10H, m), 7.84/8.22 (3-$CONH_2$, 2 × 1H, 2 × s), 8.18 (NH, 1H, s), 8.56 ppm (H5, 1H, s).

$^{13}$C NMR (DMSO-$d_6$): $\delta$ 37.52 (C5′), 41.10 (C6′), 71.99 (3′-$OCH_2$), 73.29 (2′-$OCH_2$), 81.71 (C4′), 123.09 (C2′), 127.7–128.5 ($C_6H_5$), 136.33 ($C_6H_5$), 136.73 ($C_6H_5$), 146.11 (C3), 146.83 (C5), 153.30 (C3′), 159.01 (3-CO), 167.86 ppm (C1′).

Elemental Anal. Calcd For $C_{23}H_{23}N_5O_5$: C 61.46, H 5.16, N 15.58, Found: C 61.37, H 5.17, N 15.62.

#### 4.2.5. 1-[2-(3,4-Dihydroxy-5-oxo-5*H*-furan-2-ylidene)-ethyl]-1*H*-1,2,4-triazole-3-carboxylic acid methyl ester (5)

To a solution of compound **2** (512.8 mg; 1.146 mmol) in anhydrous dichloromethane (30 mL) under the argon atmosphere, $BCl_3$ (3.2 mL; 2 × 1.6 mL) was added at −70 °C. The reaction mixture was stirred for 30 minutes. The temperature was then raised gradually to room temperature and reaction mixture stirred further one hour. Reaction was quenched by adding 10 mL of dichloromethane/methanol (1:1). The crude product was purified by silica gel column chromatography ($CH_2Cl_2$/MeOH = 20:1), to give **5** in a mixture of (*Z*)- and (*E*)-isomers (10:1) as a yellow powder (77.3 mg; 25.2%; mp = 145–148 °C); MS *m*/*z* 265.9 [M−1].

$^1$H NMR (DMSO-$d_6$): $\delta$ 3.91 ($OCH_3$, 3H, s), 5.15 (H6′, 2H, d, *J* = 14.2 Hz), 5.49 (H5′, 1H, t, *J* = 14.2 Hz), 8.83 (H5, 1H, s), 10.18 (2′-OH), 10.96 ppm (3′-OH).

$^{13}$C NMR (DMSO-$d_6$): $\delta$ 41.50 (C6′), 53.12 ($OCH_3$), 100.08 (C5′), 122.21 (C2′), 143.49 (C4′), 144.62 (C3), 145.27 (C3′), 146.98 (C5), 158.36 (3-CO), 165.11 ppm (C1′).

Elemental Anal. Calcd For $C_{10}H_9N_3O_6$: C 44.95, H 3.40, N 15.73, Found: C 45.04, H 3.41, N 15.77.

#### 4.2.6. 1-[2-(3,4-Bis-benzyloxy-5-oxo-5*H*-furan-2-ylidene)-ethyl]-1*H*-imidazole-4,5-dicarboxylic acid dimethyl ester (6)

4,5-Dimethyl-imidazole carboxylate (DMI) (1 g; 5.43 mmol) and 2,3-di-*O*-benzyl-5,6-di-*O*-acetyl-L-ascorbic acid (2.17 g; 4.98 mmol) were dissolved with stirring in dry $CH_3CN$ at room temperature. To a reaction mixture 1,8-diazobicyclo[5.4.0]undec-7-en (DBU) (2.23 mL; 14.94 mmol) was added. The temperature was lowered to 0 °C and TMSOTf (4 × 0.9 mL) was added in portions. Reaction mixture was then stirred for 24 h at 60 °C. The solvent was removed under the reduced pressure and the crude extracted with $CH_2Cl_2$ (30 mL) and saturated aq $NaHCO_3$. Organic layer was dried over $MgSO_4$, evaporated and the residue submitted to silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$ = 70:1) to yield **6** in a mixture of (*Z*)- and (*E*)-isomers (2:1) as yellow oil (705 mg; 28.06%); HRMS *m*/*z* 505.1605 [M+1].

$^1$H NMR (DMSO-$d_6$): (***Z***)-**6**: $\delta$ 3.77 ($OCH_3$, 2 × 3H, s), 5.03 (H6′, 2H, d, *J* = 7.2 Hz), 5.15 (2′-$OCH_2$, 2H, s), 5.31 (3′-$OCH_2$, 2H, s), 5.54 (H5′, 1H, t, *J* = 7.2 Hz), 7.3–7.5 (Ph, 10H+10H (***E***)-**6**, m), 7.99 ppm (H2, 1H, s). (***E***)-**6**: $\delta$ 3.72 ($OCH_3$, 3H, s), 3.77 ($OCH_3$, 3H, s), 5.14 (H6′, 2H, d, *J* = 7.6 Hz), 5.19 (2′-$OCH_2$, 2H, s), 5.41 (3′-$OCH_2$, 2H, s), 5.84 (H5′, 1H, t, *J* = 7.6 Hz), 7.3–7.5 (Ph, 10H+10H (***Z***)-**6**, m), 7.84 ppm (H5, 1H, s).

$^{13}$C NMR (DMSO-$d_6$): (***Z***)-**6**: $\delta$ 41.21 (C6′), 51.90 ($OCH_3$), 52.50 ($OCH_3$), 73.00 (3′-$OCH_2$), 73.93 (2′-$OCH_2$), 103.01 (C5′), 123.16 (C2′), 124.82 (C5), 127.8–128.8 ($C_6H_5$), 135.35 ($C_6H_5$), 135.66 (C4), 140.82 (C2), 142.74 (C4′), 147.91 (C3′), 159.84 (*C*$OOCH_3$), 162.73 (*C*$OOCH_3$), 163.51 ppm (C1′). (***E***)-**6**: $\delta$ 41.53 (C6′), 51.93 ($OCH_3$), 52.44 ($OCH_3$), 73.51 (3′-$OCH_2$), 73.88 (2′-$OCH_2$), 108.33 (C5′), 127.8–128.8 ($C_6H_5$), 135.35 ($C_6H_5$), 140.31 (C2), 142.45 (C4′), 148.62 (C3′), 159.75 (*C*$OOCH_3$), 162.76 (*C*$OOCH_3$), 163.53 ppm (C1′).

Elemental Anal. Calcd For $C_{27}H_{24}N_2O_8$: C 64.28, H 4.80, N 5.55, Found: C 64.28, H 4.81, N 5.56.

#### 4.2.7. 1-[2-(3,4-Bis-benzyloxy-2-hydroxy-5-oxo-2,5-dihydro-1*H*-pyrrol-2-yl)-ethyl]-1*H*-imidazole-4,5-dicarboxylic acid dimethyl ester (7)

To a solution of **6** (334 mg; 0.66 mmol) in dioxane (7.5 mL) and methanol (7.5 mL) ammonia (g) was introduced at 0 °C until saturation. The reaction mixture was stirred at room temperature for 24 h, then concentrated under reduced pressure and the crude product purified by silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$ = 20:1) to give **7** as a yellow oil (150 mg; 43.58%); MS *m/z* 505.1 $[M-NH_3]^+$.

$^1$H NMR (DMSO-$d_6$): $\delta$ 2.08 (H5′, 2H, m), 3.77 (C4-CO$CH_3$, 3H, s), 3.78 (C5-CO$CH_3$, 3H, s), 4.13 (H6′, 2H, m), 4.99/5.05 (2′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.1/11.1 Hz), 5.07/5.15 (3′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.7/11.7 Hz), 6.25 (OH, 1H, s), 7.3–7.4 (Ph, 10H, m), 7.85 (H2, 1H, s), 8.21 ppm (NH, 1H, s).

$^{13}$C NMR (DMSO-$d_6$): $\delta$ 37.72 (C5′), 41.96 (C6′), 51.88 (C4-CO$CH_3$), 52.44 (C5-CO$CH_3$), 72.05 (3′-$OCH_2$), 73.24 (2′-$OCH_2$), 81.59 (C4′), 123.10 (C2′), 124.16 (C5), 127.6–128.6 ($C_6H_5$), 136.15 (C4), 136.31 ($C_6H_5$), 136.70 ($C_6H_5$), 140.73 (C2), 153.14 (C3′), 159.93 (5-CO), 162.89 (4-CO), 167.86 ppm (C1′).

Elemental Anal. Calcd For $C_{27}H_{27}N_3O_8$: C 62.18, H 5.22, N 8.06, Found: C 62.29, H 5.24, N 8.08.

#### 4.2.8. 1-[2-(3,4-Bis-benzyloxy-2-hydroxy-5-oxo-2,5-dihydro-1*H*-pyrrol-2-yl)-ethyl]-1*H*-imidazole-4,5-dicarboxylic acid diamide (8)

To a solution of **6** (284 mg; 0.56 mmol) in dioxane (6 mL) and methanol (6 mL) ammonia (g) was introduced at 0 °C until saturation. The reaction mixture was stirred at room temperature for 96 h, then concentrated under reduced pressure and the crude product purified by silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$ = 20:1) to yield **8** as a yellow oil (81 mg; 29.07%); MS *m/z* 475.1 $[M-NH_3]^+$.

$^1$H NMR (DMSO-$d_6$): $\delta$ 2.10 (H5′, 2H, m), 4.39 (H6′, 2H, t, *J* = 7.0 Hz), 4.99/5.03 (2′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.1/11.1 Hz), 5.06/5.14 (3′-$OCH_2$, 2 × 1H, 2 × d, *J* = 11.9/11.9 Hz), 6.13 (OH, 1H, s), 7.3–7.4 (Ph, 10H, m), 7.59/10.67 (5-$CONH_2$, 2 × 1H, 2 × d, *J* = 2.1/2.1 Hz), 7.75 (H2, 1H, s), 7.78/7.98 (4-$CONH_2$, 2 × 1H, 2 × d, *J* = 2.0/2.0 Hz), 8.15 ppm (NH, 1H, s).

$^{13}$C NMR (DMSO-$d_6$): $\delta$ 38.06 (C5′), 42.90 (C6′), 72.00 (3′-$OCH_2$), 73.30 (2′-$OCH_2$), 81.85 (C4′), 123.05 (C2′), 126.30 (C5), 127.7–128.5 ($C_6H_5$), 135.08 (C4), 136.33 ($C_6H_5$), 136.75 ($C_6H_5$), 139.95 (C2), 153.47 (C3′), 160.44/165.72 (5-CO/4-CO), 167.94 ppm (C1′).

Elemental Anal. Calcd For $C_{25}H_{25}N_5O_6$: C 61.09, H 5.13, N 14.25, Found: C 61.25, H 5.14, N 14.25.

#### 4.2.9. 1-[2-(3,4-Bis-benzyloxy-5-oxo-5*H*-furan-2-ylidene)-ethyl]-1*H*-imidazole-4,5-dicarbonitrile (9)

4,5-Dicyano imidazole (500 mg; 4.23 mmol) and 2,3-di-*O*-benzyl-5,6-di-*O*-acetyl-L-ascorbic acid (2.17 g; 4.98 mmol) were dissolved with stirring in dry $CH_3CN$ at room temperature. To a reaction mixture DBU (1.94 mL; 12.69 mmol) was added. The temperature was lowered to 0 °C and TMSOTf (4 × 0.76 mL; 16.92 mmol) was added in portions. Reaction mixture was stirred for 24 h at 60 °C. The solvent was removed under reduced pressure and the crude product extracted with $CH_2Cl_2$ (30 mL) and sat. aq $NaHCO_3$. Organic layer was dried over $MgSO_4$ and the solvent evaporated. The residue was submitted to silica gel column chromatography ($CH_2Cl_2$/$CH_3OH$=80:1) to afford **9** in a mixture of (*Z*)- and (*E*)-isomers (4:1) as yellow oil (400 mg; 21.57%); MS *m/z* 439.1 [M+1].

$^1$H NMR (DMSO-$d_6$): (***Z***)-**9**: $\delta$ 5.09 (H6′, 2H, d, *J* = 7.3 Hz), 5.16 (2′-$OCH_2$, 2H, s), 5.31 (3′-$OCH_2$, 2H, s), 5.66 (H5′, 1H, t, *J* = 7.3 Hz), 7.3–7.4 (Ph, 10H+10H (***E***)-**9**, m), 8.34 ppm (H2, 1H, s). (***E***)-**9**: $\delta$ 5.16 (2′-$OCH_2$, 2H, s), 5.18 (H6′, 2H, d, *J* = 7.8 Hz), 5.38 (3′-$OCH_2$, 2H, s), 5.95 (H5′, 1H, t, *J* = 7.8 Hz), 7.3–7.4 (Ph, 10H+10H (***Z***)-**9**, m), 8.27 ppm (H5, 1H, s).

$^{13}$C NMR (DMSO-$d_6$): (***Z***)-**9**: $\delta$ 42.22 (C6′), 73.08 (3′-$OCH_2$), 74.00 (2′-$OCH_2$), 100.85 (C5′), 108.29 (CN), 112.41 (C5), 121.37 (C4), 123.48 (C2′), 127.8–128.8 ($C_6H_5$), 135.29 ($C_6H_5$), 135.58 ($C_6H_5$), 143.51 (C2), 143.87 (C4′), 147.73 (C3′), 163.33 ppm (C1′). (***E***)-**9**: $\delta$ 42.46 (C6′), 73.50 (3′-$OCH_2$), 73.97 (2′-$OCH_2$), 105.97 (C5′), 108.33 (CN), 112.36 (C5), 121.46 (C4), 125.08 (C2′), 127.8–128.8 ($C_6H_5$), 143.13 (C2), 143.73 (C4′), 148.41 (C3′), 163.37 ppm (C1′).

Elemental Anal. Calcd For $C_{25}H_{18}N_4O_4$: C 68.49, H 4.14, N 12.78, Found: C 68.33, H 4.15, N 12.80.

### 4.3. Biological methods

#### 4.3.1. Cell culturing

The suspension cell lines L1210 (murine lymphocytic leukaemia cells), CEM (human T-lymphocyte leukaemia cell line) were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine and 0.075% $NaHCO_3$ in a humidified atmosphere with 5% CO2 at 37 °C. Cell lines HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), Mia-PaCa-2 (pancreatic carcinoma), MCF-7 (breast epithelial adenocarcinoma, metastatic), HepG2 (hepatocellular carcinoma) and WI 38 (normal diploid human fibroblasts) were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% $CO_2$ at 37 °C.

#### 4.3.2. Proliferation assays

The panel cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at 3000 cells to 6000 cells per well according to the doubling times of specific cell line. Test agents were then added in five, 10-fold dilutions ($1 \times 10^{-8}$ to $1 \times 10^{-4}$ M) and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing in the growth medium. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in the working concentrations (DMSO concentration never exceeded 0.1%). After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay: experimentally determined absorbance values were transformed into a cell percentage growth (PG) using the formulas proposed by NIH and described previously.[16] This method directly relies on control cells behaving normally at the day of assay because it compares the growth of treated cells with the growth of untreated cells in control wells on the same plate—the results are therefore a percentile difference from the calculated expected value.

The $IC_{50}$ and $LC_{50}$ values for each compound were calculated from dose-response curves using linear regression analysis by fitting the mean test concentrations that give PG values above and below the reference value. If, however, all of the tested concentrations produce PGs exceeding the respective reference level of effect (e.g., PG value of 50) for a given cell line, the highest tested concentration is assigned as the default value (in the screening data report that default value is preceded by a '>' sign). Each test point was performed in quadruplicate in three individual experiments. The results were statistically analyzed (ANOVA, Tukey post-hoc test at $p < 0.05$). Finally, the effects of the tested substances were evaluated by plotting the mean percentage growth for each cell type in comparison to control on dose response graphs.

#### 4.3.3. Cytostatic assay

To each well of a 96-well microtiter plate were added 5–$7.5 \times 10^4$ cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (L1210) or 72 h (CEM) at 37 °C in a humidified $CO_2$-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter Counter (Coulter Electronics Ltd, Harpenden Herts, United Kingdom). The $IC_{50}$ (50%

inhibitory concentration) was defined as the concentration of compound that reduced the number of viable cells by 50%.

#### 4.3.4. Inhibition of IMP dehydrogenase by the test compounds in intact tumour cells

The activity of IMP dehydrogenase in the intact L1210 and CEM tumour cells was measured by estimation of $^3H$ release from [2,8-$^3H$]IMP that had been formed in the cells from [2,8-$^3H$]hypoxanthine in the reaction catalyzed by IMP dehydrogenase. Briefly, 300 μL of a cell suspension were mixed with 60 μL of RPMI 1640 medium or test compound and 40 μL (4 μCi) of radiolabelled Hx. At various times, 100 μL of the reaction mixture were withdrawn and mixed with 250 μL of a freshly prepared cold suspension of carbon black in 5% trichloroacetic acid. After centrifugation at 1100×*g* for 10 min, 200-μL samples of the supernatants were analyzed for radioactivity. The amount of free $^3H$ in the reaction mixture (before incubation with the cells) was less than 10% of the amount of labelled Hx added.

#### 4.3.5. Antiviral assay for hepatitis C virus

Huh 5.2 cells containing the hepatitis C virus genotype 1b I389luc-ubi-neo/NS3-3'/5.1 replicon[17] were sub-cultured in DMEM supplemented with 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin and 2% Geneticin at a ratio of 1:3 to 1:4, and grown for 3–4 days in 75 $cm^2$ tissue culture flasks. One day before addition of the compound, cells were harvested and seeded in assay medium (DMEM, 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin) at a density of 6 500 cells/well (100 μL/well) in 96-well tissue culture microtiter plates for evaluation of antimetabolic effect and CulturPlate (Perkin Elmer) for evaluation of antiviral effect. The microtiter plates were incubated overnight (37 °C, 5% $CO_2$, 95–99% relative humidity), yielding a non-confluent cell monolayer.

The evaluation of antimetabolic as well as antiviral effect of each compound was performed in parallel. Four-step, 1-to-5 compound dilution series were prepared. Following assay setup, the microtiter plates were incubated for 72 h (37 °C, 5% $CO_2$, 95–99% relative humidity). For the evaluation of antimetabolic effects, the assay medium was aspirated, replaced with 75 μL of a 5% MTS solution in phenol red-free medium and incubated for 1.5 h (37 °C, 5% $CO_2$, 95–99% relative humidity). Absorbance was measured at a wavelength of 498 nm (Safire$^2$, Tecan), and optical densities (OD values) were converted to percentage of untreated controls.

For the evaluation of antiviral effects, assay medium was aspirated and the cell monolayers were washed with PBS. The wash buffer was aspirated, and 25 μL of Glo Lysis Buffer (Promega) was added allowing for the lysis to proceed for 5 min at room temperature. Subsequently, 50 μL of Luciferase Assay System (Promega) was added, and the luciferase luminescence signal was quantified immediately (1000 ms integration time/well, Safire$^2$, Tecan). Relative luminescence units were converted into percentage of untreated controls.

The $EC_{50}$ and $EC_{90}$ (values calculated from the dose-response curve) represent the concentrations at which 50% and 90% inhibition, respectively, of viral replication is achieved. The $CC_{50}$ (value calculated from the dose-response curve) represents the concentration at which the metabolic activity of the cells is reduced by 50% as compared to untreated cells.

A concentration of compound is considered to elicit a genuine antiviral effect in the HCV replicon system when the anti-replicon effect is well above the 70% threshold at concentrations where no antimetabolic activity is observed.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.054.

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