Synthesis and Thymidine Phosphorylase Inhibition Evaluation of Pyrazolo[2,3-a]-1,3,5-triazines

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Abstract

The enzyme thymidine phosphorylase (TPase) is involved in the metabolism of thymidine. It catalyses the phosphorolysis of several nucleoside analogues serving as antiviral or antitumour agents. Besides, it also plays an important role in the angiogenesis. Thus, inhibition of TPase activity may serve as a plausible therapeutic strategy for the treatment of cancer. In this report, the structure-based design, synthesis, and anti-TPase activity of a class of novel inhibitors of TPase are described using fused bicyclic 1,3,5-triazine-2,4-dione or 1,3,5-triazine-2-thioxo-4-one as the core scaffold, and the latter scaffold shows some activity in TPase enzyme assay.

Introduction

Thymidine Phosphorylase (TPase) is an enzyme that in its normal intracellular environment catalyses the reversible phosphorolysis of pyrimidine 2'-deoxycucleosides to 2-deoxyribose-1-phosphate and their respective pyrimidine bases (Figure 1). Besides the natural 2'-deoxynucleosides, TPase also recognizes and catalyses the phosphorolysis of several nucleoside analogues that are being used clinically as antiviral and /or anti-tumour agents thus affecting the biological efficacy of these nucleoside therapeutic agents. [1] In addition, TPase is identified as the platelet-derived endothelial cell growth factor (PD-ECGF), [2] and its high expression in tumor has led to higher microvessel density (MVD), increased angiogenesis and increased metastasis.[3] [4] Therefore, inhibition of TPase activity may serve as a significant therapeutic strategy for the treatment of cancer since this may suppress the growth of tumor through inhibiting angiogenesis and at the same time it reduces the degradation of nucleoside-based anticancer agents.

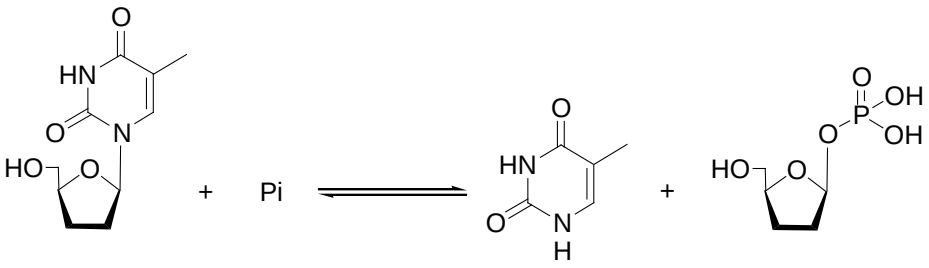


Figure 1. Reaction catalyzed by TPase.

Currently, most TPase inhibitors are monocyclic pyrimidine derivatives, and it is stressed that positions 2, 3 and 4 of the pyrimidine ring should be kept intact in order to interact with the enzyme.[5] Besides, other fused bicyclic inhibitor such as 7-deazaxanthine (7DX) (Figure 2)[6] seems to suggest that the hydrophobic pocket at the substrate binding site may be able to accommodate additional rings. In literature there are limited reports on bicyclic TPase inhibitors. Therefore, it is the aim of our lab to investigate the TPase inhibition potential of fused bicyclic 1,3,5-triazine-2,4- dione compounds and 1,3,5-triazine-2-thioxo-4-one compounds, which are close analogues of 7DX.

In this study, two series of fused bicyclic compounds were prepared, and their inhibition activity against TPase was evaluated by in vitro enzyme test.

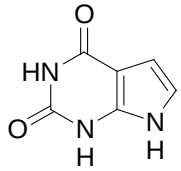
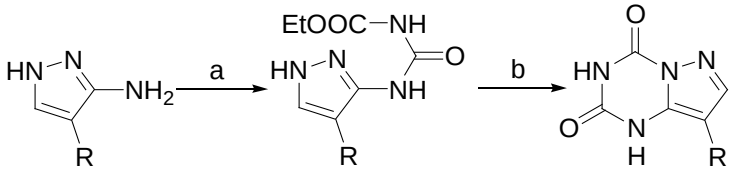


Figure 2. 7DX.

Material and methods

Chemistry

All chemicals were obtained from commercial suppliers, and used without further purification. Melting points were determined on a Gallenkamp melting point apparatus. ^{1}H NMR spectra were recorded on a Bruker DPX-300 spectrometer in DMSO-d6 and using TMS as in internal standard. The chemical processes used to prepare the fused bicyclic compounds were summarized in the following Schemes.[7, 8]

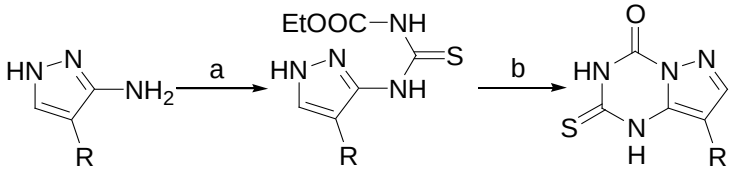


1a R=H
1b R=Cl
1c R=Br
1d R=I

2a R=H
2b R=Cl
2c R=Br
2d R=I

a: ethoxycarbonyl isocyanate, DMF, rt
b: sodium ethoxide, ethanol, reflux

Scheme1. Synthesis of 1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-diones.



1e R=H
1f R=Cl
1g R=Br
1h R=I

2e R=H
2f R=Cl
2g R=Br
2h R=I

a: ethoxycarbonyl isothiocyanate, DMF, rt
b: sodium ethoxide, ethanol, reflux

Scheme2. Synthesis of 1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2-thioxo-4-ones.

In vitro TPase enzyme assay

Enzyme and substrate were obtained from Sigma-Aldrich, and the activity assay was performed according to the manufacturer instruction, which was a classical method described by Krenitsky[9]. For IC_{50} value determination, 4 inhibitor concentrations that span over the estimated IC_{50} value were prepared and used in the assay. Inhibition rates of different concentrations were calculated and plotted against inhibitor concentrations. IC_{50} value was determined as the concentration of the inhibitor that caused 50% inhibition.

Results and discussion

Chemistry

In total eight fused bicyclic compounds (2a-2h) were prepared for in vitro TPase enzyme assay. Among these compounds, the synthesis of 2a, 2e was reported in full details previously[7]. However, synthesis of the other fused bicyclic compounds namely

2b-2d and 2f-2h was not reported, and their structure characterization was carried out (Table 2).

Table 1. Synthesis of intermediates (1b-1d, 1f-1h)

Compound	Yield(%)	Melting point(˚C)	^{1}HNMR (DMSO-d6)
1b	47%	155-156	δ1.24 (t, J = 7.0Hz, 3H, CH_3), 4.18 (q, J = 7.1 Hz, 2H, CH_2), 7.95 (s, 1H, CH), 9.36 (s, 1H, NH), 10.40 (s, 1H, NH), 12.93 (s, 1H, NH).
1c	72%	158-160	δ1.24 (t, J = 7.2Hz, 3H, CH_3), 4.18 (q, J = 7.1 Hz, 2H, CH_2), 7.96 (s, 1H, CH), 9.36 (s, 1H, NH), 10.39 (s, 1H, NH), 13.00 (s, 1H, NH).
1d	76%	166-167	δ1.25 (t, J = 7.0Hz, 3H, CH_3), 4.18 (q, J = 7.1 Hz, 2H, CH_2), 7.88 (s, 1H, CH), 9.33 (s, 1H, NH), 10.38 (s, 1H, NH), 12.99 (s, 1H, NH).
1f	64%	188-190	δ1.26 (t, J = 7.2Hz, 3H, CH_3), 4.22 (q, J = 7.1 Hz, 2H, CH_2), 7.99 (s, 1H, CH), 10.96 (s, 1H, NH), 11.45 (s, 1H, NH), 13.06 (s, 1H, NH).
1g	87%	187-189	δ1.26 (t, J = 7.0Hz, 3H, CH_3), 4.22 (q, J = 7.1 Hz, 2H, CH_2), 8.00 (s, 1H, CH), 10.95 (s, 1H, NH), 11.44 (s, 1H, NH), 13.13 (s, 1H, NH).
1h	76%	172-173	δ1.26 (t, J = 7.0Hz, 3H, CH_3), 4.22 (q, J = 7.0 Hz, 2H, CH_2), 7.93 (s, 1H, CH), 10.94 (s, 1H, NH), 11.41 (s, 1H, NH), 13.11 (s, 1H, NH).

Table 2. Synthesis of fused bicyclic compounds (2b-2d, 2f-2h)

Compound	Yield(%)	Melting point(˚C)	^{1}HNMR (DMSO-d6)
2b	53%	>300	δ7.94 (s, 1H, CH), 11.72 (s, 1H, NH), 12.33 (s, 1H, NH).
2c	66%	>300	δ7.91 (s, 1H, CH), 11.72 (s, 1H, NH), 12.25 (s, 1H, NH).
2d	61%	292-294	δ7.82 (s, 1H, CH), 11.67 (s, 1H, NH), 12.01 (s, 1H, NH).
2f	46%	258-260	δ8.02 (s, 1H, CH), 12.80 (s, 1H, NH), 13.79 (s, 1H, NH).
2g	68%	>300	δ8.00 (s, 1H, CH), 12.78 (s, 1H, NH), 13.70 (s, 1H, NH).
2h	61%	199-201	δ7.92 (s, 1H, CH), 12.77 (s, 1H, NH), 13.50 (s, 1H, NH).

In vitro TPase enzyme assay

Table 3. Inhibitory effect of compounds **2a-2h** on recombinant human TPase

Compound	*R*	*IC_{50}[a](μM)*	*Compound*	*R*	*IC_{50}[a](μM)*
2a	H	>150	2e	H	75±9.2
2b	Cl	>150	2f	Cl	98±12.7
2c	Br	>150	2g	Br	95±0
2d	I	>150	2h	I	108±8.5
7DX		42±3.5			

[a]A 1mM concentration of dThd was used as the substrate for the TP reactions. This assay tested at least four concentrations of each compound. Values represent means of minimum two experiments (standard deviations in parentheses).

Based on the enzyme test results (Table 3), IC_{50} values of 1H,3H-pyrazolo[1,5-a]-1,3,5-triazine-2,4-diones (**2a-2d**) were above 150 μM, and it seems that the replacement of uracil ring with 1, 3, 5-triazine ring has made the compounds become non-active. However, with bioisosteric replacement of oxygen with sulfur at position 2 of the triazine ring, the pyrazolo[1,5-a]-1,3,5-triazine-2-thioxo-4(1H,3H)-ones (**2e-**

2h) showed some activity. Therefore, this bioisosteric replacement would be necessary for the activity of compounds bearing pyrazolo[1,5-a]-1,3,5-triazine ring.

In addition, compared with compound **2e**, IC_{50} values of compounds **2f-2h** were larger, which means different halogen substitutions at position 8 of the pyrazole ring decrease the activity. Since halogen atoms are hydrophobic, it is possible that the environment surrounding that position is hydrophilic, and replacement of halogen atoms with some hydrophilic group might improve the activity.

In conclusion, 1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2-thioxo-4-ones possess better activity than 1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-diones, but they are less potent compared with 7DX. Further, different halogen substitutions at position 8 of the pyrazole ring could decrease the activity.

General experimental procedure

Chemistry

General method for preparation of Carbamic acid, [(1H-pyrazol-3-ylamino) carbonyl]-, ethyl esters (1a-1d)

To the fine suspension of the corresponding amines (1 mmol) in anhydrous DMF (3ml), ethoxycarbonyl isocyanate (1mmol) was added. After stirring the mixture for 2h at room temperature, cold water (30ml) was added. The precipitated product was filtered, washed with cold water and recrystallized from acetonitrile.

General method for preparation of 1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-diones (2a-2d)

1mmol corresponding Carbamic acid, [(1H-pyrazol-3-ylamino) carbonyl]-, ethyl ester was refluxed in a mixture of 4ml ethanol and 1ml 21%wt EtONa ethanol solution for 1h. Precipitate was collected by filtration, then dissolved in water and acidified with HCI until pH 4. Precipitate coming out was filtered and recrystallized from methanol.

1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-dione(2a)

Yield: 26%. Mp: >300°C (lit [7]: 330˚C). ESI-MS 152.9 m/z (M+1).^{1}HNMR (DMSO-d6): δ5.79 (d, 1H, CH, *J*=1.63Hz), 7.78 (d, 1H, CH, *J*=1.50Hz), 11.58 (s, 1H, NH), 11.88 (s, 1H, NH).

1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-dione, 8-chloro- (2b)

Yield: 53%. Mp: >300°C. ESI -MS 187.0, 189.0 m/z (M+1). ^{1}HNMR (DMSO-d6): δ7.94 (s, 1H, CH), 11.72 (s, 1H, NH), 12.33 (s, 1H, NH)..

1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-dione, 8-bromo- (2c)

Yield: 66%. Mp: >300°C. ESI -MS 231.0, 233.0 m/z (M+1). ^{1}HNMR (DMSO-d6): δ7.91 (s, 1H, CH), 11.72 (s, 1H, NH), 12.25 (s, 1H, NH)..

1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2,4-dione, 8-iodo- (2d)

Yield: 61%. Mp: 292-294˚C. ESI-MS 278.9 m/z (M+1). ^{1}HNMR (DMSO-d6): δ7.82 (s, 1H, CH), 11.67 (s, 1H, NH), 12.01 (s, 1H, NH).

General method for preparation of Carbamic acid, N-[(1H-pyrazol-3-ylamino) thioxomethyl]-, ethyl esters (1e-1h)

To the fine suspension of the corresponding amines (1 mmol) in anhydrous DMF (3ml), ethoxycarbonyl isothiocyanate (1mmol) was added. After stirring the mixture for 2h at room temperature, cold water (30ml) was added. The precipitated product was filtered, washed with cold water and recrystallized from ethanol.

General method for preparation of 1,3-dihydro-pyrazolo[2,3-a]-1,3,5-triazine-2-thioxo-4-ones (2e-2h)

1mmol corresponding Carbamic acid, N-[(1H-pyrazol-3-ylamino) thioxomethyl]-, ethyl ester was refluxed in a mixture of 4ml ethanol and 1ml 21%wt EtONa ethanol solution for 1h. Precipitate was collected by filtration, then dissolved in water and acidified with HCI until pH 4. Precipitate coming out was filtered and recrystallized from methanol.

Pyrazolo[2,3-a]-1,3,5-triazin-4(1H)-one, 2,3-dihydro-2-thioxo- (2e)

Yield: 54%. Mp: 284-286˚C (lit[7]: 298-300˚C). ESI-MS 169.0 m/z (M+1). ^{1}HNMR (DMSO-d6): δ5.90 (d, 1H, CH, *J*=1.68Hz), 7.88 (d, 1H, CH, *J*=1.65Hz), 12.73 (s, 1H, NH), 13.45 (s, 1H, NH).

Pyrazolo[2,3-a]-1,3,5-triazin-4(1H)-one, 8-chloro -2,3-dihydro-2-thioxo-(2f)

Yield: 46%. Mp: 258-260˚C. ESI-MS 202.9, 205 m/z (M+1). ^{1}HNMR (DMSO-d6): δ8.02 (s, 1H, CH), 12.80 (s, 1H, NH), 13.79 (s, 1H, NH).

Pyrazolo[2,3-a]-1,3,5-triazin-4(1H)-one, 8-bromo-2,3-dihydro-2-thioxo-(2g)

Yield: 68%. Mp: >300°C. ESI -MS 246.9, 249.0 m/z (M+1). ^{1}HNMR (DMSO-d6): δ8.00 (s, 1H, CH), 12.78 (s, 1H, NH), 13.70 (s, 1H, NH).

Pyrazolo[2,3-a]-1,3,5-triazin-4(1H)-one, 8-iodo-2,3-dihydro-2-thioxo-(2h)

Yield: 61%. Mp: 199-201˚C. ESI-MS 294.8 m/z (M+1). ^{1}HNMR (DMSO-d6): δ7.92 (s, 1H, CH), 12.77 (s, 1H, NH), 13.50 (s, 1H, NH).

In vitro TPase enzyme assay

For all compounds, inhibition rate of each concentration used in IC_{50} determination was obtained by the following procedure:

To 980μl 1mM Thymidine phosphate buffer solution (pH 7.4) in the absorption cuvette was added 10μl compound DMSO solution (for blank, add in 10μl DMSO), and the absorption value at 290 nm was recorded. Then 10μl 500unit/ml enzyme phosphate buffer solution (pH 7.0) was added in, and absorption values at 10seconds, 15seconds, 20seconds were recorded successively. These absorption values were plotted against time, and linear regression was performed to obtain the initial velocity of that concentration, which was equaled to the slope of the line.

Then inhibition rate of that concentration was calculated using the following format:

$$\text{Inhibition rate} = \frac{\textit{Initial velocity of that concentration} - \textit{Initial velocity of blank}}{\textit{Initial velocity of blank}} \times 100\%$$

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