Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 3287–3290

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF 5-SUBSTITUTED QUINAZOLINONE DERIVATIVES AND THEIR INHIBITORY ACTIVITY *IN VITRO*

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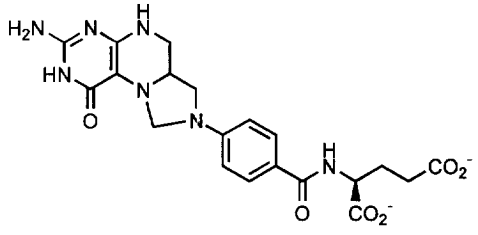
Received 29 July 1998; accepted 6 October 1998

Abstract: Quinazolinone derivatives **I** and their methyl esters were synthesized and evaluated as nonclassical lipophilic inhibitors of thymidylate synthase. Compounds **Ib** and **Ic** containing OH and CO_2H as R substituents, respectively, were most effective, indicating that hydrogen bonding may contribute to the increased inhibitory activity. These compounds further showed high cytotoxic activity against tumor cells in culture.

Introduction: Thymidylate synthase (TS; EC 2.1.1.45) is involved in the catalytic reaction of deoxyuridylate (dUMP) to deoxythymidylate (dTMP), which is a one-carbon transfer to the 5'-position of uridylate. In this process N^5,N^{10}-methylenetetrahydrofolate (**1**) plays a critical role as a cofactor, and this folate derivative delivers one-carbon as a methylene unit from serine to uridylate.[1]



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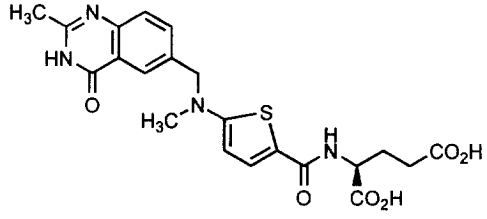

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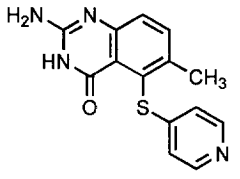
Since DNA contains thymine as a base component instead of uracil, rapidly proliferating cells require an abundant supply of deoxythymidylate for the biosynthesis of DNA. For the synthesis of dTMP the aforementioned reaction is the sole *de novo* pathway, and, without an exogenous supply of thymidine, blockade of this step by the inhibition of TS would lead to a "thymineless cell death" ultimately.

For this purpose many classical antifolates are under development as antitumor agents. They are structurally similar to the natural folates containing L-glutamic acid moiety in the molecule, and one example is ZD1694 (Tomudex, **2**).[2,3] The L-glutamic acid component is essential for active transport into cells *via* reduced folate uptake systems and for the binding of antifolate to the active site of TS through hydrogen bonding of α- and γ-carboxylates of antifolates to basic amino acid residues of the enzyme.[4] The binding affinity is further increased through polyglutamation of classical antifolates catalyzed by folylpolyglutamate synthetase.[2] This polyglutamation produces noneffluxing poly-γ-glutamates which lead to longer retention of these agents inside cells for higher cytotoxic activity.[5] However, these classical folate analogues are implicated in two detrimental features: (1) drug resistance which is originated from the defective cell transport by mutation, and (2) toxicity to the host which is due to unnecessarily long retention inside *normal* cells. One

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00602-7

way to overcome these implications is to delete or modify L-glutamic acid component from the folate analogues, making these analogues nonclassical lipophilic inhibitors of TS. Recently 2-amino-6-methyl-5-(pyridin-4-ylsulfanyl)-3*H*-quinazolin-4-one (**3**)[6,7] has been reported as a nonclassical inhibitor of human and *E. coli* TS with the inhibitory binding constants (K_i) of 15 and 49 nM, respectively. This compound further showed high cytotoxic activity against tumor cells in culture.



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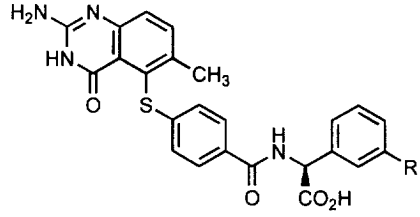
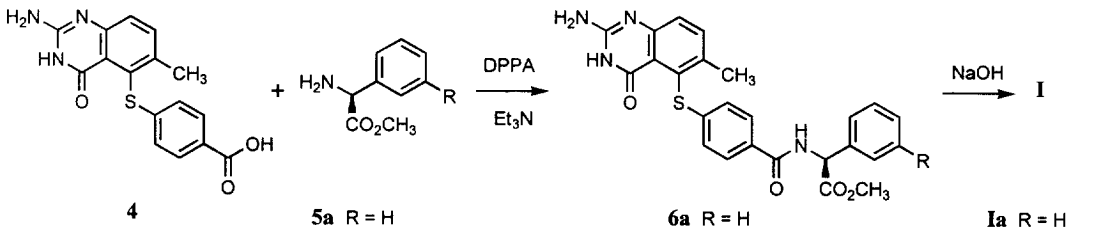


I

In this report 5-arylthio-substituted quinazolinone derivatives **I** were designed as TS inhibitors based on the bicyclic ring system of **3**, and phenylglycines were introduced to take advantage of both classical and nonclassical antifolates. The compounds containing phenylglycines are different from those containing glutamic acid in two aspects: (1) they can overcome the abovementioned glutamate-related resistance and toxicity, and (2) they are more lipophilic for passive transport into cells by diffusion. These analogs still have one acid at the α-position, and increased binding affinity at the active site is expected. This paper is made up of the synthesis of quinazolinone compounds and the evaluation of these compounds for inhibition against TS and for cytotoxic growth inhibition of several tumor cell lines of murine and human origin *in vitro*.

Chemistry: The synthesis of 5-arylthio-substituted quinazolinone derivatives **I** was performed according to Scheme I. 4-[(2-Amino-6-methyl-4-oxo-3,4-dihydroquinazolin-5-yl)sulfanyl]benzoic acid **4**[6] was reacted with properly substituted (*S*)-2-phenylglycine methyl esters **5** in the presence of diphenylphosphoryl azide (DPPA) to form amide esters **6**, which were converted to **I** on hydrolysis.[8]

Scheme I



4

5a R = H
5b R = OH
5c R = CO_2CH_3

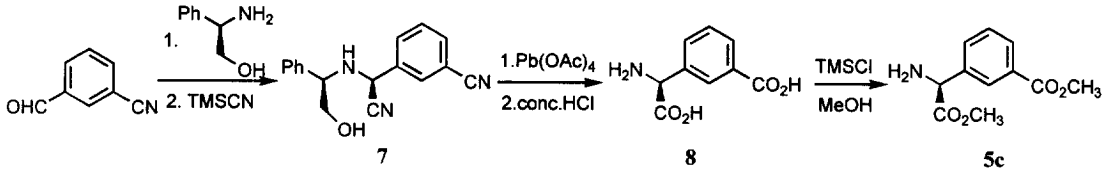
6a R = H
6b R = OH
6c R = CO_2CH_3

Ia R = H
Ib R = OH
Ic R = CO_2H

Optically active phenylglycine methyl esters **5b** and **5c** were easily prepared in 5 steps from benzaldehydes by diastereoselective Strecker synthesis using 2-phenylglycinol as chiral auxiliary,[9] and the synthesis of **5c** is shown in Scheme II as an example. 3-Cyanobenzaldehyde was reacted with (*R*)-2-phenylglycinol to form Schiff base, and trimethylsilyl cyanide was added *in situ* to lead to the stereospecific addition of cyanide to the Schiff base. (*S*)-α-amino nitrile **7** was obtained as a major isomer, which was separated by

column chromatography. This amino nitrile was hydrolyzed to generate amino acid **8** preceded by removal of auxiliary by oxidative cleavage with lead tetraacetate. Finally, acid **8** was esterified to form dimethyl ester **5c**.

Scheme II



7 8 5c

Biological Activity: Compounds **6a-c** and **Ia-c** were tested as inhibitors of bacterial and human TS and as inhibitors of the growth of four tumor cell lines: L1210 (mouse lymphocytic leukemia), LY3.7.2C TK-/- (mouse lymphoma, thymidine kinase deficient), CCRF-CEM (human leukemia), and HT-29 (human colon adenocarcinoma) as shown in Table 1. The values of relative potency of the inhibition against *Lactobacillus casei* and human TS were all equal or greater than 1.0 compared with compound **3**, and highest relative value was 15.7. The inhibitory activities of carboxylic acids were compared with those of corresponding esters, and acids **Ia-c** were found to be 1.6 to 5.0-fold more potent than esters **6a-c**. These data indicate that phenylglycine moieties connected to 5-arylthio-quinazolinone increase the inhibitory binding affinity of quinazolinone **3** against the target enzyme, and free α-carboxyl group is required as a hydrogen donor for stronger binding and

Table 1. Thymidylate Synthase and Cell Growth Inhibition Data for Compounds **6a-c** and **Ia-c** in Comparison with Compound **3**

		relative potency[a] of TS inhibition		cell growth inhibition[f] (IC_{50}, μM)			
compound	R	*L. casei*[b]	human[c]	L1210	LY TK-/-	CCRF-CEM	HT-29
6a	H	1.0	2.2	0.4	0.5	0.2	0.6
Ia	H	5.0 (5.0)	ND	2.0	0.8	5.4	>20
6b	OH	2.5	2.7	2.0	0.3	1.5	8.4
Ib	OH	4.1 (1.6)	13.5 (5.0)	0.7	0.6	3.4	17
6c	CO_2CH_3	1.0	4.5	0.7	0.7	0.2	0.6
Ic	CO_2H	3.3 (3.3)	15.7 (3.5)	0.5	0.7	0.05	1.2
3		1.0[d]	1.0[e]	1.1	1.5	0.8	3.7

[a] Defined as IC_{50}(**3**)/IC_{50}(compound) determined in the same test. Numbers in parentheses indicate the comparison of potency of compound **I** against compound **6**. ND = not determined. [b] TS inhibition assay was done at six concentrations ranging from 0.3 to 10 mM to calculate IC_{50}. Enzyme activity was measured by following the change in UV absorbance at 340 nm in an assay solution containing 2.5 mM dUMP and 3 mM methylenetetrahydrofolate. [c] Enzyme activity was measured by the tritium release method[10] using 25 μM [5-^{3}H]dUMP and 300 μM methylenetetrahydrofolate over a range of compound concentration from 0.3 to 100 nM. [d] IC_{50}(**3**) = 1.0 ~ 4.9 μM. [e] IC_{50}(**3**) = 42 ~ 69 nM. [f] MTT colorimetric assay[11] was used to determine IC_{50}. Cells were seeded at 6,000 (LY TK-/-, HT-29) or 10,000 (L1210, CCRF-CEM) cells per well in 96-well plates, and growth was measured spectrophotometrically over a range of concentrations following a 3-day (L1210, LY TK-/-) or 4-day (CCRF-CEM, HT-29) incubation in RPMI-1640 medium containing 5% fetal calf serum and 50 μg/ml penicillin/streptomycin. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide.

thus for better inhibition. Most effective were compounds **Ib** and **Ic** where there are hydroxyl and carboxyl groups as additional hydrogen donors at the meta position of phenyl ring of phenylglycine, and this high potency is assumed to be arisen from the extra hydrogen bonding for the stronger binding interaction.

These six compounds also showed similar or better cytotoxicity against tumor cell lines in comparison with compound **3** (Table 1). In particular, submicromolar cell growth inhibition was observed in compounds **6a**, **6c**, and **Ic**, and it is believed that increased binding affinity and/or better cell permeability may contribute to this high cytotoxic activity.

Currently antitumor activity is being tested *in vivo* in BDF_1 mice against LY3.7.2C TK-/- cell line implanted i.m. following i.p. administration of test compounds. Interim results showed that disodium salt of compound **Ic** cured tumor in mice at 120 mg/kg twice daily for 10 days, and the details of these results will be presented elsewhere.

Acknowledgment: We would like to thank Profs. D. V. Santi (University of California, San Francisco) and I.-Y. Kwak (Pai Chai University, Korea) for the generous gift of human and *L. casei* TS plasmids, respectively, and Prof. H. H. Evans (Case Western Reserve University) for the thankful supply of LY3.7.2C TK-/- cell line.

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7. The development of AG337, dihydrochloride salt of **3**, by Agouron Pharmaceuticals has been discontinued after phase III clinical trials.
8. **Ia**: mp 228.7 - 229.8 °C; IR (KBr) 3400, 1705, 1595, 1475, 1380, 1290 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.30 (s, 3H), 5.56 (d, 1H, *J* = 7.53 Hz), 6.88 (br, 2H), 6.97 (d, 2H, *J* = 8.29 Hz), 7.30 ~ 7.37 (m, 5H), 7.47 (d, 2H, *J* = 6.41 Hz), 7.61 (d, 1H, *J* = 8.66 Hz), 7.74 (d, 2H, *J* = 8.63 Hz), 8.89 (d, 1H, *J* = 7.53 Hz); Mass m/z 461 (M+1).
   Ib: IR (KBr) 3380, 3220, 1720, 1480, 1300 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.33 (s, 3H), 5.45 (d, *J* = 7.31 Hz, 1H), 6.73 (dd, *J* = 7.78, 1.57 Hz, 1H), 6.88 (d, *J* = 6.23 Hz, 2H), 7.01 (d, *J* = 8.47 Hz, 2H), 7.16 (t, *J* = 9.17 Hz, 1H), 7.43 (d, *J* = 8.39 Hz, 1H), 7.75 (t, *J* = 10.24 Hz, 3H), 8.87 (d, *J* = 7.40 Hz, 1H), 9.48 (s, 1H); Mass m/z 499 (M + Na); Anal. calculated for $C_{24}H_{20}N_4O_5S$ HCl $2H_2O$ C: 52.50, H: 4.58, N: 10.20, S: 5.84; found C: 52.84, H: 4.34, N: 10.35, S: 5.82.
   Ic: mp 286 - 287 °C; IR (KBr) 3400, 1715, 1610, 1490, 1305 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.29 (s, 3H), 5.65 (d, *J* = 7.3 Hz, 1H), 6.47 (br, 2H), 6.96 (d, *J* = 8.4 Hz, 2H), 7.26 (d, *J* = 8.4 Hz, 1H), 7.49 (t, 3H), 7.57 (d, *J* = 8.4 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 3H), 7.89 (d, *J* = 7.8 Hz, 1H), 8.05 (s, 1H), 9.01 (d, *J* = 7.5 Hz, 1H); Mass m/z 504 (M^+); Anal. calculated for $C_{25}H_{20}N_4O_6S$ $2H_2O$ C: 55.55, H: 4.48, N: 10.36, O: 23.68, S: 5.93; found C: 55.52, H: 4.14, N: 10.30, O: 24.00, S: 6.04.
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