Med Chem Res (2009) 18:495–510
DOI 10.1007/s00044-008-9144-x

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Novel furoxan NO-donor pemetrexed derivatives: design, synthesis, and preliminary biological evaluation

Tao Min · Bixin Yi · Peng Zhang · Jun Liu · Can Zhang · Huiping Zhou

Received: 5 December 2007 / Accepted: 25 November 2008 / Published online: 23 December 2008

Abstract Design and synthesis of a new class of potent hybrid compounds obtained by joining NO-donor furoxanyl moieties, through an appropriate spacer arm, to the pemetrexed segment, which was prepared in a modified simple route, are presented. Structures of all the target compounds were characterized by using Fourier-transform infrared (FT-IR), ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and electrospray ionization mass spectroscopy (ESI-MS). In addition, preliminary evaluation of antitumor activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay based on four different cancer cell lines (BGC-1, HL60-1, SMMC-1, and A549-1) was performed. The results suggested that different length of spacer arm in the hybrid compounds did have an impact on the molecules' capability to inhibit cancer cell growth to various degrees, but without showing significant difference on regular pharmacological behaviors. Among all the synthesized hybrids, compound **1f** showed the strongest inhibitory activity against all the tested cell lines and it is currently under our further investigation.

Keywords Medicinal chemistry · Drug design · Hybrid · Furoxans · Pemetrexed

Tao Min and Bixin Yi contributed equally to this work.

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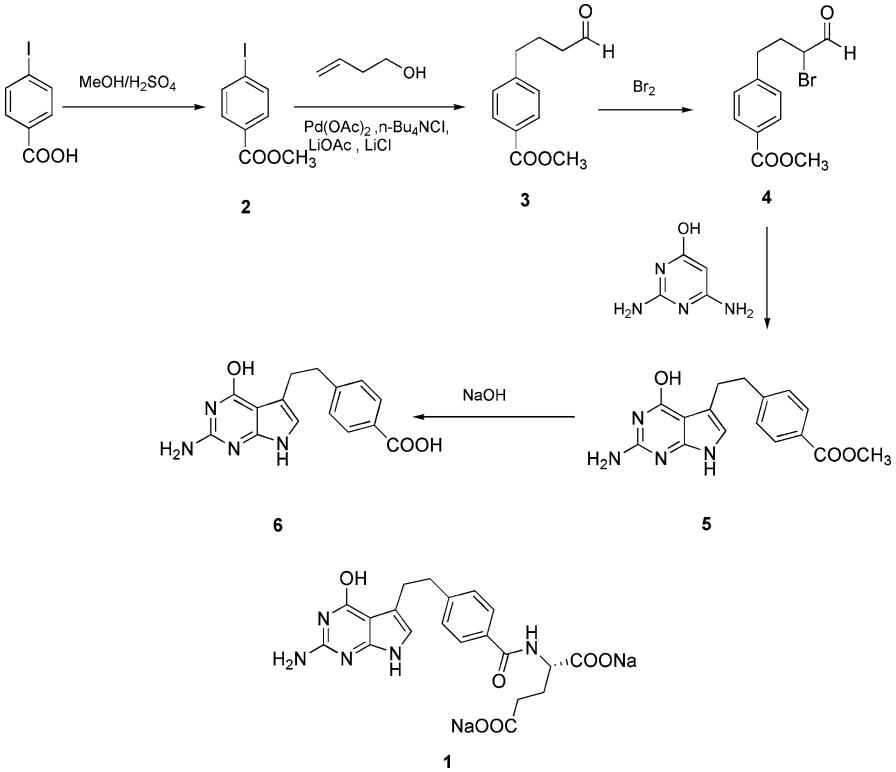
Introduction

Since the discovery of the capability of aminopterin to induce remission in children with acute leukemia several decades ago, folate-dependent pathways have been extensively explored as key targets in the development of new and effective anticancer agents (Farber *et al*., 1974). Research into the development of antifolates as antitumor agents has been actively pursued since the 1950s and has led to a number of successful antifolates to date, including methotrexate, 5-fluorouracil, and raltitrexed, which are now widely used in the treatment of various solid and hematologic malignancies (Paz-Ares *et al*., 2003). In 1992, a report by Taylor et al. described the discovery of pemetrexed disodium (**1**; Scheme 1), a multitargeted folate analogue that suppresses tumor growth by impeding both DNA synthesis and folate metabolism (Green, 2002). Further studies identified that it inhibits several folate-dependent enzymes involved in the de novo biosynthesis of thymidine and purine nucleotides, including thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycinamide ribonucleotide formyltransferase (GARFT). Due to its favorable preclinical profiles, this agent has entered the clinical trials, showing encouraging activities in numerous tumors, such as non-small-cell lung carcinoma (NSCLC), malignant mesothelioma, carcinomas of the breast, etc. Many studies



Scheme 1 Structure of pemetrexed disodium and synthesis of the key intermediate **6**

with this drug, as a single agent or in combination with some other agents, are currently ongoing (Adjei, 2000, 2004; Postmus, 2002).

As promising an agent as it is, there are still several unsatisfactory aspects about pemetrexed. For instance, it is very susceptible to oxidation degradation and pH must be adjusted to a proper value just before pemetrexed disodium is applied in the clinical stage (Zhang and Trissel, 2006). Therefore, more investigation is in an urgent need to overcome these shortcomings.

Medicinal chemical hybridization is a well-developed approach in the drug design process. It involves the combination of two complementary biological activities by joining appropriate pharmacophoric groups directly or via spacers (Christiaans and Timmerman, 1996). Based on this idea, there is widespread interest in the design of hybrid drugs in which an appropriate pharmacophoric group is linked with a nitric oxide (NO) releasing moiety (Wang *et al*., 2002; Sorba *et al*., 2003). It is known that furoxan derivatives are able to activate the soluble guanylate cyclase by releasing NO under the action of thiol cofactor (Civelli *et al*., 1994; Ferioli *et al*., 1995; Medana *et al*., 1994). Since NO is considered to be involved in many bioregulatory processes including the influence on the tumor cell growth, the furoxan moieties could be employed in the design of a variety of hybrid molecules with interesting potential for treating a variety of diseases (Kerwin and Heller, 1994).

The main purpose of this study is to develop a new type of pemetrexed derivatives containing NO-donating furoxanyl moieties in the hope of more potent antitumor activities through both antifolate- and NO-mediated cellular mechanism. Additionally, the synthetic route of the pemetrexed segment was modified for much simpler application. More importantly, novel structures with furoxanyl moieties leading to better water solubility were designed and prepared. The effect of different length of spacer arms in the conjugation between pemetrexed and furoxan moieties on the molecule's capability to exert its NO-releasing property and the anticancer activity were preliminarily probed.

Results and discussion

Design and synthesis

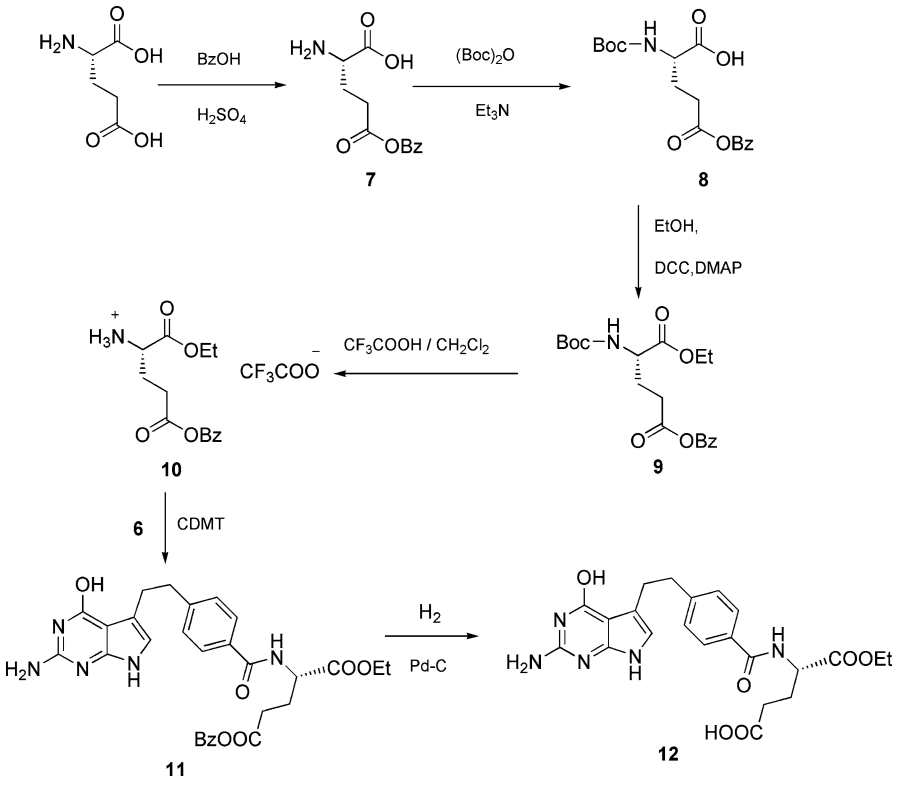
Preparation of the target compounds **1a–h** could be generally divided into four sections by a sequence involving synthesis of the intermediate **6**, the pemetrexed segment **12**, different furoxanyl moieties **16a–h** and the final products **1a–h**.

As described in Scheme 1, esterification of 4-iodobenzoic acid with methanol by using concentrated sulfuric acid as the catalyst led to 1-(4-iodophenyl)ethanone **2**, which was treated with 3-buten-1-ol via palladium-catalyzed coupling reaction in the presence of $Pd(OAc)_2$, LiOAc, LiCl, and n-Bu_4NCl to give 4-(4-acetylphenyl) butanal **3** by a previous method (Larock *et al.*, 1989). After chromatographic purification, bromination of compound **3** with bromide was performed to afford 4-(4-acetylphenyl)-2-bromobutanal **4**, which was, without further purification, condensed with 2,4-diamino-6-hydroxypyrimidine in the presence of sodium

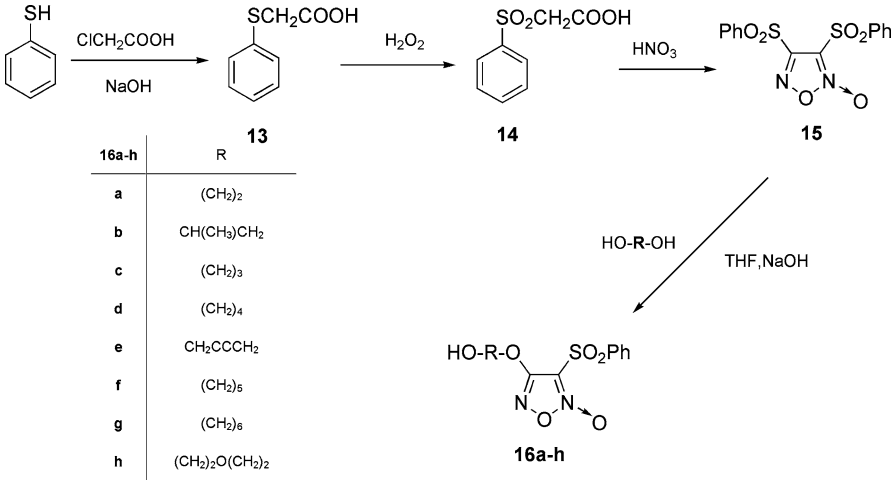
acetate in the mixed solvents of water and methanol, followed by standard hydrolyzation with NaOH, finally furnishing the key intermediate **6**.

Preparation of the pemetrexed segment **12** followed the synthetic route illustrated in Scheme 2. Compound **10** was obtained using the reported method (Sanda *et al.*, 2001) with some minor modification, starting from L-glutamic acid, followed by the protection of the γ-hydroxyl group with benzyl alcohol in the presence of H_2SO_4, the Boc-protection of the amino group using triethylamine, esterification with ethanol in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylamino-pyridine (DMAP), and finally acidification with a dichloromethane solution of trifluoroacetic acid (TFA). The resulting product was then condensed with compound **6** catalyzed by 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT), followed by hydrogenation with the palladium on carbon catalyst in order to achieve the deprotection of benzyl function, finally yielding the pemetrexed segment **12**.

The furoxanyl moieties **16a–h** were synthesized according to the route presented in Scheme 3. Esterification of the starting thiophenol with $ClCH_2COOH$ in basic medium resulted in compound **13**, which was converted to the important intermediate **15** by oxidation with H_2O_2 and cyclization with fuming nitric acid using one-pot method (Xu *et al.*, 2002). The latter products were left to react with different chain lengths of diol derivatives HO-R-OH to give a series of furoxan NO-donors **16a–h**.



Scheme 2 Synthesis of the pemetrexed segment **12**

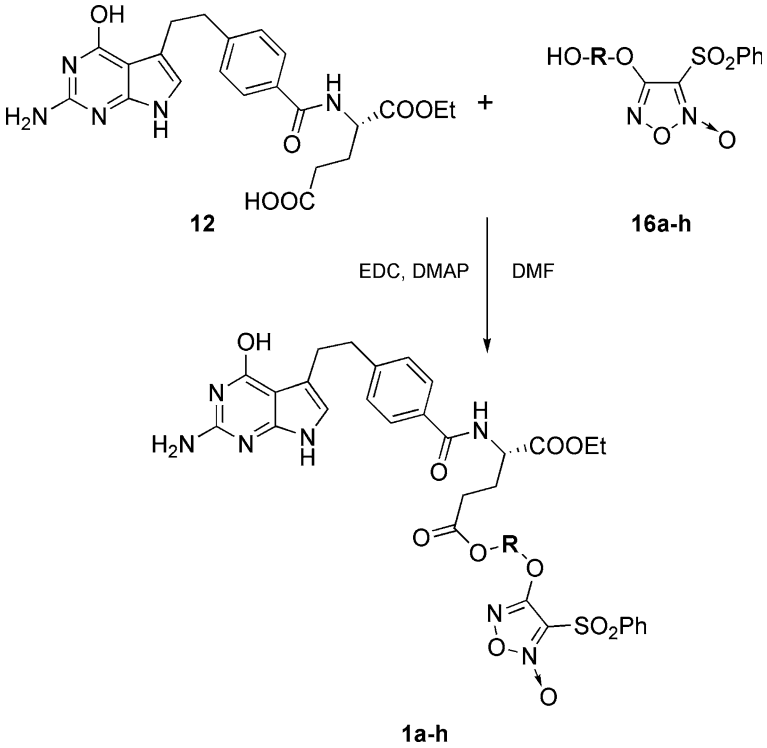


Scheme 3 Synthesis of the furoxanylmoieties **16a–h**

As depicted in Scheme 4, the target compounds **1a–h** were given by the condensation between the pemetrexed segment **12** and the furoxanyl moieties **16a–h** in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and DMAP in dimethyl formamide (DMF) medium.

During our synthetic route, the Heck reaction from compound **2** to **3** generated three isomers, which were difficult to isolate from one another. In addition, compound **3** is not stable enough for storage and is very prone to polymerization. Thus, as soon as compound **3** was separated, it was rapidly put into use for the next reaction. To synthesize the intermediates **16a–h**, large excess of diol should be employed because they were inclined to form diether byproducts. Thus, the ideal amount of diol should be enhanced to more than 5 equivalents. In the process of constructing compound **11**, it was difficult to separate the small amount of byproduct, which was formed during the condensation between **6** and CDMT, from the product compound **11** due to their similar molecular weight and polarity. Several chromatography eluent such as petroleum/acetoacetate, methanol/acetone, *n*-butanol/glacial acetic acid/water, and chloroform/methanol had been tried, with the chloroform/methanol system in a proper ratio achieving a relatively satisfactory result.

Synthesis of the target structures was first catalyzed by DCC, resulting in an unknown compound with molecular weight 695.5 as assessed by ESI mass analysis. Considering that the DCC catalyst was not rapidly separated from the reactant after finishing catalysis due to too tight binding, the unknown structure perhaps was proposed to be the conjugate containing the DCC catalyst. Interestingly, when using another catalyst (EDC) that has much stronger catalyzing ability than DCC, this phenomenon did not occur. This could be explained by that, due to its insufficient catalyzing ability or relatively larger stereospecific blockade of DCC, the corresponding intermediate was not able to perform reaction completely, resulting in the above DCC conjugates remaining.



Scheme 4 Synthesis of the target compounds **1a–h**

Biological evaluation

As a preliminary experiment, all the target compounds were evaluated for in vitro antitumor activity by using the MTT assay based on four different cancer cell lines: human lung carcinoma cell A549, human poorly differentiated gastric adenocarcinoma cell BGC-823, human hepatoma carcinoma cell SMMC-7721, and human promyelocyte leukemic cell HL-60. The results are summarized in Table 1. The findings showed that all the synthesized compounds exhibited weak inhibitory activity against A549-1, except compound **1f**. Compounds **1b**, **1d**, **1e**, **1f**, and **1h** were able to inhibit BGC-1 cell growth at a compound concentration of 1×10^{-5} moL L^{-1}, showing high inhibiting activity similar to that of the parent pemetrexed. Notably, compound **1f** was outstanding from the remaining ones in that it exhibited the most efficient inhibitory capability. All the molecules **1a–h** synthesized possessed good bioactivity against HL-60 cell growth. In this case, compound **1f** and the control pemetrexed were of the same order of magnitude, with compound **1f** being slightly more potent compared with the pharmacophore pemetrexed. The enhanced bioactivity suggested that the introduction of the furoxan group, which releases NO, did play a cooperative role in the exertion of antitumor activity, leading to a better balance between NO- and pemetrexed-dependent activity in the hybrid. However, in the case of the SMMC-1 cell line, the sensitivity

Table 1 In vitro cytotoxicity study of the target compounds and the control pemetrexed

Compound	C [10^{-6} mol L^{-1}]	BGC	HL60	SMMC	A549
1	0.1	−	+	−	−
	1	−	++	−	−
	10	+	++	−	−
	0.1	−	−	−	−
1a	1	−	−	−	−
	10	−	+	−	−
	0.1	−	−	−	−
1b	1	−	++	−	−
	10	++	++	−	−
	0.1	−	−	−	−
1c	1	−	−	−	−
	10	−	+	−	−
	0.1	−	−	−	−
1d	1	−	+	−	−
	10	++	+	++	−
	0.1	−	−	−	−
1e	1	−	−	−	−
	10	+	++	+	−
	0.1		+	−	−
1f	1	+	++	−	−
	10	+	++	−	+
	0.1	−	−	−	−
1g	1	−	−	−	−
	10	+	++	++	−
	0.1	−	−	−	−
1h	1	−	−	−	−
	10	−	++	−	−

The symbols ++, +, and – indicate >80%, 50–80%, and <50% antitumor inhibition activities in each assay, respectively

of compound **1f** was not satisfactory. In contrast, compounds **1d**, **1e**, and **1g** were observed to exhibit inhibitory activity with medium intensity. According to these cytotoxicity studies, it is difficult to investigate the exact relationship between the molecules' biological activities and the chain lengths of the used spacers. Much work remains to be done to sort out the structure–activity relationships (SARs), and further investigation is currently ongoing in our laboratory to continue these studies.

Conclusion

In this study, a new type of hybrid molecules, which may be expected to possess both NO- and pemetrexed-dependent bioactivities, has been designed, synthesized,

and evaluated for preliminary in vitro cytotoxicity studies. Chemical modification of the pemetrexed pharmacophore by the introduction of furoxan group gave biological activities of the same order of magnitude as that of the parent pemetrexed. Among all the target compounds, compound **1f** seemed slightly more potent than the native pemetrexed towards the four tested cell lines (BGC-1, HL60-1, SMMC-1, and A549-1) and is worthy of further investigation. According to the obtained data, the effect of chain length of spacer arm on the molecules' bioactivity as well as the structure–activity relationships have not been clearly revealed. Therefore, further studies are warranted to investigate in vitro NO release from the hybrid molecules, in vivo cytotoxicity, and clearer structure–activity relationship studies, which are being considered and ongoing in our group.

Experimental section

Synthesis and characterization

Melting points were determined in open capillaries and are reported uncorrected. IR spectra were recorded on Fourier-transform infrared spectrometer (Nicolet 2000) in KBr discs.^{1}H-NMR and ^{13}C-NMR spectra were obtained for solutions in dimethyl sulfoxide (DMSO)-d_6 or $CDCl_3$ or D_2O using a Bruker (AVACE) AV-500 spectrometer. Mass spectra (ESI (+) 70 V) were performed on Agilent 1100-MS spectrometer. Solvents and reagents were analytical grade or higher, distilled and dried by standard methods whenever required.

Preparation of the intermediate **6** for pemetrexed moiety

*1-(4-Iodophenyl)ethanone (**2**)*

A mixture of 4-iodobenzoic acid (105 g, 42 mmol), methanol (400 mL), and concentrated sulfuric acid (15 mL) was heated under reflux for 3 h. The mixture, cooled at room temperature, was concentrated under reduced pressure, followed by the treatment of equal volume (100 mL) of water and CH_2Cl_2. The aqueous phase was extracted with CH_2Cl_2, which was combined with the previous CH_2Cl_2 layer. The organic phase was washed with aqueous Na_2CO_3 (3 × 100 mL) and water (3 × 100 mL), dried over Na_2SO_4, and evaporated to obtain **2** as a white lamellar powder solid (96 g, 85%). M.p. 113–117°C.

*4-(4-Acetylphenyl)butanal (**3**)*

A mixture of 1-(4-iodophenyl) ethanone **2** (66 g, 0.25 mol), LiOAc (28.7 g, 0.43 mol), LiCl (32.3 g, 0.76 mol), *n*-Bu_4NCl (37.5 g, 0.13 mol), Pd $(OAc)_2$ (1.42 g, 6.3 mmol), 3-buten-1-ol (27 mL, 0.31 mol), and DMF (600 mL) was stirred at 70°C for 12 h. After cooling at room temperature, water (600 mL) and CH_2Cl_2 (600 mL) were added into the resulting mixture. The CH_2Cl_2 layer was obtained and the aqueous phase, which was extracted with CH_2Cl_2 (2 × 500 mL),

was then combined with the previous CH_2Cl_2 layer. Washed with water (3 × 900 mL), the organic phase was concentrated to give a black oil, which was purified on a silica-gel column (petroleum ether/acetic ether, v/v, 6:1) to afford **3** as a pale yellow oil (22.0 g, 43%). ^{1}H-NMR (DMSO-d_6), (δ ppm): 1.82 (m, 2H, $CH_2CH_2CH_2CHO$), 2.43 (d, 2H, J = 7.4 Hz, $CH_2CH_2CH_2CHO$), 2.65 (t, 2H, J = 7.7 Hz, $CH_2CH_2CH_2CHO$), 3.80 (s, 3H, $COCH_3$), 7.30 (d, 2H, J = 8.5 Hz, ArH), 7.86 (d, 2H, J = 8.5 Hz, ArH), 9.75 (t, 1H, J = 1.5 Hz, CHO).

*4-(4-Acetylphenyl)-2-bromobutanal (**4**)*

To a solution of compound **3** (20.6 g, 0.1 mol) in acetic acid (300 mL) was added bromine (0.2 mol, 10.2 mL) dropwise. The mixture was then stirred for 1 h, poured into water (500 mL), which was extracted with acetic ether (3 × 200 mL). The combined organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 150 mL) and water (2 × 150 mL), dried over Na_2SO_4, and concentrated to give **4** as an amber oil (23.2 g, 81.4%), which was used immediately for the next reaction.

*4-[2-(2-Amino-4,7-dihydro-4-oxo-1H-pyrrolo [2,3-d] pyrimidin-5-yl)ethyl] benzoic acid methyl ester (**5**)*

To a solution of 2,4-diamino-6-hydroxypyrimidine (5.0 g, 0.04 mol) in water (85 mL) and methanol (85 mL) was added compound **4** (8.5 g, 0.03 mol), followed by addition of sodium acetate (4.9 g, 0.06 mol). The mixture was stirred at 45°C for 3 h, cooled to room temperature, and filtered under vacuum to afford an amber solid 5 (7.9 g, 84.9%). ^{1}H-NMR (DMSO-d_6), (δ ppm): 2.84 (m, 2H, CH_2CH_2), 2.93 (m, 2H, CH_2CH_2), 3.80 (s, 3H, $COOCH_3$), 5.97 (s, 2H, H_2 N), 6.30 (d, 1H, J = 2.0 Hz, NHCH=), 7.33 (d, 2H, J = 8.2 Hz, ArH), 7.85 (d, 2H, J = 8.2 Hz, ArH), 10.58 (s, 1H, NH).

*4-[2-(2-Amino-4,7-dihydro-4-oxo-1H-pyrrolo [2,3-d] pyrimidin-5-yl)ethyl] benzoic acid (**6**)*

A mixture of compound **5** (4.2 g, 13.5 mol) and 2 N NaOH (52 mL) was stirred at 45°C for 2 h, cooled to room temperature, and filtered. The red filtrate was acidified with 6 N HCl to afford a white precipitate, which was washed with water and dried under vacuum to give **6** as a off-white solid (3.7 g, 92.2%). ^{1}H-NMR (DMSO-d_6), (δ ppm): 2.45 (m, 2H, CH_2CH_2), 2.91 (m, 2H, CH_2CH_2), 5.99 (s, 2H, H_2N), 6.27 (d, 1H, J = 2.1 Hz, NHCH=), 7.27 (d, 2H, J = 8.2 Hz, ArH), 7.79 (d, 2H, J = 8.2 Hz, ArH), 10.59 (s, 1H, NH).

Preparation of the pemetrexed segment (**12**)

*γ-Benzyl-L-glutamic acid (**7**)*

To a mixture of diethyl ether (125 mL) containing concentrated sulfuric acid (12.5 mL) was added benzyl alcohol (500 mL). Ether was evaporated and

L-glutamic acid (18.4 g, 5.13 mol) was added in small portions. The mixture was stirred at room temperature for 20 h and diluted with ethanol (250 mL), and pyridine (62.5 mL) was added under vigorous stirring. The mixture was cooled to 0°C and left overnight at this temperature. The resulting precipitate was filtered and washed with anhydrous ether. Recrystallization from boiling water containing 10% of pyridine afforded **7** as a white solid powder (14 g, 51%). M.p. 181–182°C.

*N-tert-Butoxycarbonyl-γ-benzyl-L-glutamic acid (**8**)*

To a solution of compound **7** (14 g, 59 mmol) in DMF and H_2O solution (280 mL, v/v, 1:1) were added di-*tert*-butyl dicarbonate (13.0 g, 59.6 mmol) and triethylamine (6.1 g, 59.5 mmol) at 0°C, and the resulting mixture was stirred at room temperature for 12 h. Evaporation under vacuum afforded an oily residue, which was suspended in ethyl acetate, acidified to pH 2 with aqueous 1 N HCl, and extracted with ethyl acetate (2 × 200 mL). The extract was washed with H_2O (100 mL) and dried over Na_2SO_4. Evaporation of the solvent afforded **8** as a colorless oil (18.5 g, 91%).

*N-tert-Butoxycarbonyl-α-ethyl-γ-benzyl-L-glutamate (**9**)*

A mixture of compound **8** (18.5 g, 55 mmol), DCC (13.3 g, 63 mmol), DMAP (2.0 g, 16 mmol), anhydrous ethanol (10 mL), and CH_2Cl_2 (60 mL) was stirred at room temperature overnight. The resulting mixture was filtered and the filtrate was concentrated under vacuum to obtain a white mealiness solid **9** (15 g, 75%). M.p. 64–65°C.

*α-ethyl-γ-benzyl-L-glutamate·CF_3COOH (**10**)*

To a solution of compound **9** (12 g, 33 mmol) in CH_2Cl_2 (100 mL) was added trifluoroacetic acid (37.5 mL) at 0°C, and the reaction was stirred at room temperature for 12 h. The mixture was then concentrated to give a solid which was washed with diethyl ether, affording **10** as a white needle solid (10.5 g, 80%).

*N-[4-[2-(2-Amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d]-pyrimidin-5-yl)ethyl]benzoyl]-γ-benzyl-L-glutamic acid ethyl ester (**11**)*

To an ice-cooled suspension of compound **6** (4 g, 7 mmol) in DMF (40 mL) was added *N*-methylmorpholine (5 mL), followed by the addition of 2-chloro-4, 6-dimethoxy-1,3,5-triazine (CDMT) (3.2 g, 9.4 mmol). The resulting mixture was stirred for 2 h at 25°C, poured into water (100 mL), and extracted with CH_2Cl_2 (3 × 80 mL). The organic phase was washed with water (3 × 100 mL), dried over Na_2SO_4, and evaporated to give a residue, which was purified by a silica-gel column chromatography. The final product **11** appeared as a white solid powder (3.2 g, 95%). MS (ESI (+) 70 V) m/z: 580.3([M + $Cl]^-$, base peak).

*N-[4-[2-(2-Amino-4,7-dihydro-4-oxo-1H-pyrrolo [2,3-d]-pyrimidin-5-yl)ethyl] benzoyl]-α-ethyl-L-glutamic acid (**12**)*

Compound **11** (3 g, 5.5 mmol) and 0.3 g (8 wt % equiv) of 10% palladium on carbon catalyst were added to DMF (40 mL). Hydrogenation of the mixture was carried out at normal pressure for 8 h. After filtration of the mixture, the filtrate was concentrated and purified by silica-gel column chromatography to obtain a light green prism solid **12** (2.2 g, 88%). M.p. 201–204°C; MS (ESI (+) 70 V) m/z: 454.1 ($[M]^-$, basepeak); ^{1}H-NMR (DMSO-d6), (δ ppm): 10.9 (s, ^{1}H, CONH(cycle)), 10.7 (s, 1H, CONH(chain)), 8.6 (d, 1H, NHCH=, 7.79), (m, 2H, J = 8.2 Hz, ArH), 7.29 (m, 2H, J = 8.2 Hz, ArH), 6.64 (d, 2H, NH_2), 6.37 (s, 1H, J = 2.0 Hz, NHCH=), 4.43 (m, 1H, CONHCH), 4.09 (m, 2H, OCH_2), 2.97 (t, 2H, CH_2CH_2), 2.86 (t, 2H, CH_2CH_2), 2.06 (m, 2H, CH_2CH_2COOH), 1.97 (m, 2H, CH_2CH_2COOH), 1.18 (t, 3H, CH_3).

Preparation of the furoxanyl moieties (**16a–h**)

*2-(phenylthio) acetic acid (**13**)*

To a solution of benzenethiol (24.2 g, 0.22 mol) and NaOH (8.8 g, 0.22 mol) in 95 % ethanol was added slowly a mixture of $ClCH_2COOH$ (22.7 g, 0.24 mol) and Na_2CO_3 (12.7 g, 0.12 mol) in water (200 mL). After stirring at room temperature for 3 h, the mixture was refluxed for another 1 h, cooled to room temperature, and acidified with 6 N HCl to pH 2. Removal of ethanol and filtration gave **13** as a white rod-shape crystal (34 g, 92%). M.p. 161–163°C.

*3,4-Bis(phenylsulfonyl)-1,2,5-oxadizazole-oxide (**15**)*

Aqueous H_2O_2 (30%, 16.2 mL, 0.16 mol) was added to a solution of compound **13** (13.4 g, 80 mmol) in glacial acetic acid (65 mL). After 2.5 h of stirring at room temperature, the mixture turned clean. Fuming nitric acid (95%, 32 mL, 0.72 mol) was added and the stirring was continued at 90°C for 1 h. After the mixture was cooled completely, it was collected by filtration and dried under vacuum to give **15** as a white needle solid (11.1 g, 76%). M.p. 154–156°C.

General procedure for the preparation of compounds (**16a–h**)

To a solution of compound **15** (1.83 g, 5 mmol) in DMF (40 mL) was added HO-R-OH (10 mmol) at about 5°C, followed by dropwise addition of 2.5 N aqueous NaOH (4 mL) and a 30 min stirring. The solution was concentrated under vacuum at room temperature. The residue was treated with EtOAc and the resulting mixture was washed twice with water. Aqueous phases were extracted with EtOAc. The combined organic layers were dried, and evaporated under vacuum to give a residue that was purified by silica-gel column chromatography.

General procedure for the preparation of the targeted compounds (**1a**–**h**)

A mixture of compound **12** (0.2 g, 0.44 mmol), **16a**–**h** (0.52 mmol), 1-ethyl-3-(3-dimethyl a minopropyl)-carbodiimide hydrochloride (EDC) (0.1 g, 0.52 mmol), a catalytic amount of DMAP, and DMF (5 mL) was stirred for 24 h at room temperature. The resulting mixture was poured into water (20 mL), which was extracted with CH_2Cl_2 (3 × 20 mL). The CH_2Cl_2 layer was washed with water (3 × 20 mL), concentrated, and purified by silica-gel column chromatography to obtain the product as an amber solid.

Data of the targeted compounds **1a**–**h**

*Compound **1a***

Yield 69%; M.p. 101–105°C; ^{1}H-NMR(DMSO-d6), (δ ppm): 10.5 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.63 (d, 1H, NHCH=), 7.99 (m, 2H, J = 8.2 Hz, ArH), 7.82 (m, 1H, J = 8.2 Hz, ArH), 7.77 (t, 2H, J = 8.1 Hz, ArH), 7.70 (t, 2H, J = 8.1 Hz, ArH), 7.27 (t, 2H, J = 8.1 Hz, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 4.59 (t, 2H, OCH_2), 4.48 (m, 1H, CH), 4.39 (t, 2H, CH_2), 4.10 (m, 2H, $CO_2CH_2CH_3$), 2.97 (t, 2H, CH_2CH_2), 2.86 (t, 2H, CH_2CH_2), 2.15(m, 1H, CH), 2.04 (m, 1H, CH), 1.19 (t, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.63, 151.24, 152.19, 159.27 (Pyridin), 98.71, 113.34 (Pyrrolo), 127.39, 128.27, 146.23 (Py), 171.63, 171.66, 171.94 (–CO–), 14.10 ($COOCH_2CH_3$), 51.87 (–CH–), 128.55, 129.97, 135.53, 137.87 ($PhSO_2$), 110.32, 158.68 (Furox), 64.34, 68.56 (Glycol); IR (KBr, cm^{-1}): 3402, 245, 931, 2864, 2792, 1739, 1664, 1627, 1548, 1500, 1446, 1164, 595.96; MS (ESI (+) 70 V) *m/z*: 724.2 ([M] $^+$, base peak).

*Compound **1b***

Yield 52%; M.p. 88–91°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.6 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.60 (d, 1H, NHCH=), 7.99 (m, 2H, J = 8.2 Hz, ArH), 7.87 (m, 1H, J = 8.2 Hz, ArH), 7.72 (t, 2H, J = 8.1 Hz, ArH), 7.70 (t, 2H, J = 8.1 Hz, ArH), 7.27 (t, 2H, J = 8.1 Hz, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 5.22 (m, 1H, CH), 4.50 (m, 2H, CH_2), 4.36 (s, 2H, OCH_2), 4.09 (m, 2H, OCH_2 × 2), 2.97 (t, 2H, CH_2), 2.85 (t, 2H, CH_2), 2.46 (m, 2H, CH_2), 2.12 (m, 1H, CH), 2.02 (m, 1H, CH), 1.37 (d, 2H, CH_3), 1.18 (s, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.60, 151.26, 152.13, 159.21 (Pyridin), 98.68, 113.34 (Pyrrolo), 127.33, 128.23, 146.25 (Py), 171.65, 171.69, 171.91 (–CO–), 13.97 ($COOCH_2CH_3$), 51.82 (–CH–), 128.26, 129.93, 136.02, 137.22 ($PhSO_2$), 110.30, 158.13 (Furox), 15.56, 67.40, 72.33 (1, 3-Propylene Glycol); IR (KBr, cm^{-1}): 3342, 3220, 2998, 2931, 2792, 1737, 1664, 1622, 1546, 1500, 1446, 1352, 1209, 1166; MS (ESI (+) 70 V) *m/z*: 738.2 ([M + Cl]$^-$, base peak).

*Compound **1c***

Yield 63%; M.p. 114–117°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.5 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.60(d, 1H, NHCH=), 8.00 (d, 2H, J = 8.2 Hz, ArH), 7.87 (t, 1H, J = 8.1 Hz, ArH), 7.74 (m, 4H, J = 8.1 HZ, ArH), 7.27 (d, 2H, J = 8.2 Hz, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 4.45 (m, 3H, CH_2, CH), 4.12(m, 4H, OCH_2*2), 2.97 (2H, t, CH_2,), 2.86 (2H, t, CH_2), 4.7 (2H, d, 2H), 2.07 (4H, $CH_2 \times 2$), 1.18 (t, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.69, 151.28, 152.20, 159.23 (Pyridin), 98.72, 113.32 (Pyrrolo), 127.40, 128.27, 146.22 (Py), 171.63, 171.66, 171.91 (–CO–), 14.16 ($COOCH_2CH_3$), 51.89 (–CH–), 128.57, 129.92, 135.54, 137.90 ($PhSO_2$), 110.31, 158.69 (Furox), 62.02, 28.15, 65.94 (Propylene Glycol); IR (KBr, cm^{-1}): 3334, 3218, 927, 2856, 1735, 1664, 1618, 1548, 1498, 1448, 1375, 1353, 1164; MS (ESI (+) 70 V) *m/z*: 772.4 ($[M + Cl]^-$, base peak).

*Compound **1d***

Yield 64%; M.p. 109–112°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.5 (s, 1H, CONH(cycle)),10.1 (s, 1H, CONH(chain)), 8.6 (d, 1H, NHCH=), 8.00 (d, 2H, J = 8.2 Hz, ArH), 7.87 (t, 1H, J = 8.1 Hz, ArH), 7.75 (m, 4H, J = 8.1 HZ, ArH), 7.28 (d, 2H, J = 8.2 Hz, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 4.45 (m, 1H, CH), 4.41 (t, 2H, OCH_2), 4.09 (t, 4H, $OCH_2 \times 2$), 2.97 (t, 2H, CH_2), 2.85 (t, 2H, CH_2), 2.48 (t, 2H, CH_2), 2.12 (m, 1H, CH), 2.01 (m, 1H, CH), 1.80 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 1.18 (t, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.60, 151.29, 152.14, 159.23 (Pyridin), 98.70, 113.37 (Pyrrolo), 127.40, 128.23, 146.25 (Py), 171.63, 171.65, 171.91 (–CO–), 14.19 ($COOCH_2CH_3$), 51.83 (–CH–), 128.53, 129.91, 135.58, 137.90 ($PhSO_2$), 110.40, 158.64 (Furox), 24.93, 25.76, 65.34, 68.32 (Butanediol); IR (KBr, cm^{-1}): 3328, 3220, 2952, 2935, 2856, 1731, 1662, 1620, 1552, 1498, 1448, 1371, 1257, 1166; MS (ESI (+) 70 V) *m/z*: 786.3 ($[M + Cl]^-$, base peak).

*Compound **1e***

Yield 67%; M.p. 84–86°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.5 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.60 (d, 1H, NHCH=), 8.01 (d, 2H, J = 8.2 Hz, ArH), 7.88 (t, 1H, J = 8.1 Hz, ArH), 7.75 (m, 4H, J = 8.1 HZ, ArH), 7.28 (d, 2H, J = 8.2 Hz, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 5.23 (t, 2H, OCH_2), 4.82 (d, 2H, OCH_2), 4.43 (m, 1H, CH), 4.10 (m, 1H, OCH_2), 2.97 (t, 2H, CH_2), 2.85 (t, 2H, CH_2), 2.13 (m, 1H, CH), 2.02 (m, 1H, CH), 1.20 (m, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.59, 151.27, 152.12, 159.21 (Pyridin), 98.69, 113.35 (Pyrrolo), 127.35, 128.29, 146.26 (Py), 171.51, 171.68 (–CO–), 14.01 ($COOCH_2CH_3$), 51.87 (–CH–), 128.29, 129.99, 136.15, 137.94 ($PhSO_2$), 110.61, 157.96 (Furox), 51.69, 51.87, 83.99 (Alkyne); IR (KBr, cm^{-1}): 3357, 3224, 2925, 2854, 2792, 1739, 1660, 1618, 1544, 1502, 1450, 1359, 1296, 1166; MS (ESI (+) 70 V) *m/z*: 782.1 ($M + Cl]^-$, base peak).

*Compound **1f***

Yield 45%; M.p. 92–94°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.6 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.60 (d, 1H, NHCH=), 7.99 (d, 2H, J = 8.2 Hz, ArH), 7.87 (t, 1H, J = 8.1 Hz, ArH), 7.75 (m, 4H, J = 8.1 HZ, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 4.43 (m, 1H, CH), 4.36 (s, 2H, OCH_2), 4.07 (m, 4H, $OCH_2 \times 2$), 2.97 (t, 2H, CH_2), 2.85 (t, 2H, CH_2), 2.45 (t, 2H, CH_2), 2.11 (m, 1H, CH), 2.00 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 1.61 (m, 2H, CH_2), 1.38 (m, 2H, CH_2), 1.18 (m, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.60, 151.29, 152.15, 159.23 (Pyridin), 98.70, 113.36 (Pyrrolo), 127.35, 128.25, 146.28 (Py), 172.07, 171.72 (–CO–), 14.00 ($COOCH_2CH_3$), 51.84 (–CH–), 128.25, 129.92, 136.02, 137.18 ($PhSO_2$), 110.41, 158.63 (Furox), 25.64, 27.94, 30.03, 60.51, 61.34, 69.31 (Pentanediol); IR (KBr, cm^{-1}): 3353, 3313, 3220, 3136, 3026, 2943, 2869, 1731, 1643, 1614, 1550, 1502, 1450, 1371, 1298, 1255,1164; MS (ESI (+) 70 V) *m/z*: 800.3 ($[M + Cl]^-$, base peak).

*Compound **1g***

Yield 62%; M.p. 144–166°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.6 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.60 (d, 1H, NHCH=), 7.99 (d, 2H, J = 8.2 Hz, ArH), 7.87 (t, 1H, J = 8.1 Hz, ArH), 7.75 (m, 4H, J = 8.1 HZ, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 4.44 (m, 1H, CH), 4.36 (s, 2H, OCH_2), 4.06 (m, 4H, $OCH_2 \times 2$), 2.97 (t, 2H, CH2), 2.85 (t, 2H, CH_2), 2.46 (m, 2H, CH_2), 2.11 (m, 2H, CH), 2.02 (m, 2H, CH), 1.72 (s, 2H, CH_2), 1.57 (s, 2H, CH_2), 1.34 (s, 4H, $CH_2 \times 2$), 1.18 (s, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.63, 151.23, 152.17, 159.29 (Pyridin), 113.32, 98.75 (Pyrrolo), 146.22, 128.27, 127.38 (Py), 171.71, 172.05 (–CO–), 14.01 ($COOCH_2CH_3$), 51.88 (–CH–), 128.22, 129.91, 136.06, 137.19 ($PhSO_2$), 110.42, 158.67 (Furox), 63.23, 28.12, 25.67, 25.59, 29.78, 68.43 (Hexanediol); IR (KBr, cm^{-1}): 3332, 3226, 2935, 2796, 1728, 1664, 1621, 1552, 1502, 1450, 1373, 1257, 1166; MS (ESI (+) 70 V) *m/z*: 814.4 ($[M + Cl]^-$, base peak).

*Compound **1h***

Yield 44%; M.p.122–124°C; ^{1}H-NMR (DMSO-d6), (δ ppm): 10.6 (s, 1H, CONH(cycle)), 10.1 (s, 1H, CONH(chain)), 8.60 (d, 1H, NHCH=), 7.99 (d, 2H, J = 8.2 Hz, ArH), 7.87 (t, 1H, J = 8.1 Hz, ArH), 7.75 (m, 4H, J = 8.1 HZ, ArH), 6.30 (s, 1H, J = 2.0 Hz, NHCH=), 5.97 (s, 2H, NH_2), 4.50 (t, 2H, OCH_2), 4.43 (m, 1H, CH), 4.16 (t, 2H, OCH_2), 4.09 (m, 2H, OCH_2), 3.79 (m, 2H, OCH_2), 3.68 (m, 2H, OCH_2), 2.97 (t, 2H, CH_2), 2.85 (t, 2H, CH_2), 2.46 (m, 2H, CH_2), 2.11 (m, 2H, CH), 2.02 (m, 2H, CH), 1.20 (t, 3H, CH_3); ^{13}C-NMR (DMSO-d6), (δ ppm): 117.66, 151.26, 152.13, 159.22 (Pyridin), 98.72, 113.37 (Pyrrolo), 127.40, 128.29, 146.26 (Py), 171.74, 172.06 (–CO–), 14.00 ($COOCH_2CH_3$), 51.83 (–CH–), 128.26, 129.93, 136.09, 137.17 ($PhSO_2$), 110.46, 158.63 (Furox), 64.65, 69.78, 69.84, 69.95 (Diglycol); IR (KBr, cm^{-1}): 3338, 3220, 2933, 2869, 1735, 1643, 1618, 1548, 1500, 1446, 1355, 1257, 1164; MS (ESI (+) 70 V) *m/z*: 802.2 ($[M + Cl]^-$, base peak).

Biological evaluation

MTT assay was performed to study the in vitro cytotoxicity of the target compounds based on four cell lines: human lung carcinoma cell A549, human poorly differentiated gastric adenocarcinoma cell BGC-823, human hepatoma carcinoma cell SMMC-7721, and human promyelocyte leukemic cell HL-60 by using pemetrexed disodium, which was synthesized by our group using a modified approach, as the control article. The detailed experiment procedures were as follows: to a bottle of well-developed cells in the exponential growth phase was added 0.25% Trypsin solution to make sure the adherent cells to shed, affording the cell suspension (2–4 $\times$ 10^4 cells/mL counted). The cell suspension (180 μL) was inoculated to each well of a 96-well cell culture board and placed in a humidified 37°C, 5% CO_2 incubator. The fluid was changed after the cell adhesion for 24 h, and the tested drug was added (20 μL/well), and incubated for 48 h. MTT was added to the 96-well assay plate (20 μL/well), followed by the reaction in the incubator for 4 h. After removing the supernatant, DMSO (150 μL) was added to each well and was vibrated gently on a swing bed for 5 min. The absorption values of 96-well were detected on the enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm, and the corresponding cell inhibition rate (CI%) was calculated by the equation: CI% = (OD value from the negative group − OD value from the positive group)/OD value from the positive group $\times$ 100%, where OD = optical density.

Acknowledgements The authors are grateful to Professor Wenbin Shen and Professor Longsheng Shen for recording the NMR spectra and MS spectra. This study is financially supported by National Natural Science Foundation of China (30772662), Program for New Century Excellent Talents in University (NCET-06-0499), Ministry of Education key project (107062), and 111 Project from the Ministry of Education of China and the State Administration of Foreign Expert Affairs of China (No. 111-2-07).

References

Adjei AA (2000) Pemetrexed: a multitargeted antifolate agent with promising activity in solid tumors. Ann Oncol 11:1335–1341. doi:10.1023/A:1008379101017

Adjei AA (2004) Pemetrexed (ALIMTA), a novel multitargeted antineoplastic agent. Clin Cancer Res 10:4276s–4280s. doi:10.1158/1078-0432.CCR-040010

Christiaans JAM, Timmerman H (1996) Cardiovascular hybrid drugs: combination of more than one pharmacological property in one single molecule. Eur J Pharm Sci 4:1–22. doi:10.1016/0928-0987(95)00029-1

Civelli M, Caruso P, Giossi M, Bergamaschi M, Razzetti R, Bongrani S, Gasco A (1994) CHF 2206, a new potent vasodilating and antiaggregating drug as potential nitric oxide donor. Eur J Pharmacol 255:17–24. doi:10.1016/0014-2999(94)90077-9

Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff JA (1974) Temporary remissions in acute leukemia in children produced by folic acid antagonist...aminopterin. CA Cancer J Clin 24:297–305

Ferioli R, Folco GC, Ferretti C, Gasco AM, Medana M, Fruttero R, Civelli RM, Gasco A (1995) A new class of furoxan derivatives as NO donors: mechanism of action and biological activity. Br J Pharmacol 114:816–820

Green MR (2002) Alimta (pemetrexed disodium): a multitargeted antifolate for the treatment of mesothelioma Lung Cancer. Lung Cancer 38:55–57. doi:10.1016/S0169-5002(02)00359-8

Kerwin JF, Heller M (1994) The arginine-nitric oxide pathway: a target for new drugs. Med Res Rev 14:23–74. doi:10.1002/med.2610140103

Larock RC, Leung WY, Stolz-Dunn S (1989) Synthesis of aryl-substituted aldehydes and ketones via palladium-catalyzed coupling of aryl halides and non-allylic unsaturated alcohols. Tetrahedron Lett 30:6629–6632. doi:10.1016/S0040-4039(00)70636-8

Medana C, Ermondi G, Fruttero R (1994) Furoxans as nitric oxide donors. 4-Phenyl-3-furoxancarbonitrile: thiol-mediated nitric oxide release and biological evaluation. J Med Chem 37:4412–4416. doi:10.1021/jm00051a020

Paz-Ares L, Bezares S, Tabernero JM, Castellanos D, Cortes-Funes H (2003) Review of a promising new agent–pemetrexed disodium. Cancer 97:2056–2063. doi:10.1002/cncr.11279

Postmus PE (2002) Activity of pemetrexed (alimta), a new antifolate, against non-small cell lung cancer. Lung Cancer 38:3–7. doi:10.1016/S0169-5002(02)00350-1

Sanda F, Fujiyama T, Endo T (2001) Chemical synthesis of poly-glutamic acid by polycondensation of -glutamic acid dimer: synthesis and reaction of poly-glutamic acid methyl ester. J Polym Sci Pol Chem 39:732–741. doi :10.1002/1099-0518(20010301)39:5<732::AID-POLA1045>3.0.CO;2-P

Sorba G, Galli U, Cena C (2003) A new furoxan NO-donor rabeprazole derivative and related compounds. Chembiochem 4:899–903. doi:10.1002/cbic.200300617

Wang PG, Xian M, Tang X, Wu X, Wen Z, Cai T, Janczuk AJ (2002) Nitric oxide donors: chemical activities and biological applications. Chem Rev 102:1091–1134. doi:10.1021/cr000040l

Xu X, Wang W, Zhang Y (2002) Improved synthesis of 3,4-Bis(phenylsulfonyl)-1,2,5-Oxadizole-2-Oxide. J China Pharm U 33:548–549

Zhang Y, Trissel LA (2006) physical and chemical stability of pemetrexed in infusion solutions. Ann Pharmacother 40:1082–1085. doi:10.1345/aph.1G715