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# Quinoxaline chemistry. Part 11. 3-Phenyl-2[phenoxy- and phenoxymethyl]-6(7) or 6,8-substituted quinoxalines and *N*-[4-(6(7)-substituted or 6,8-disubstituted-3-phenylquinoxalin-2-yl)hydroxy or hydroxymethyl]benzoylglutamates. Synthesis and evaluation of in vitro anticancer activity and enzymatic inhibitory activity against dihydrofolate reductase and thymidylate synthase

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**Abstract**

Twenty-four out of twenty-nine quinoxalines were selected at the National Cancer Institute, Bethesda, Md, USA, for in vitro anticancer screening. Among these, 10 derivatives exhibited high values of percent tumor growth inhibition at a concentration of $10^{-4}$ M in all cancer cell lines. Four of these compounds maintained these values at $10^{-5}$ M, whereas a certain number exhibited significant values of percent inhibition at the most diluted concentrations ($10^{-8}$–$10^{-6}$ M). Inhibitory activity against dihydrofolate reductase (DHFR) (bovine and rat liver) was determined for the most active compounds. This test showed that this type of quinoxaline exhibited an appreciable activity in comparison with the previously described aza analogues. In the other test (*Lactobacillus casei*, thymidylate synthase (TS), human HTS) no or poor activity was detected in both series of compounds. 

*Keywords:* Antifolate agents; Anticancer agents; Enzyme inhibition; Dihydrofolate reductase

## 1. Introduction

In our research program on quinoxaline derivatives acting as in vitro anticancer compounds, we have described so far more than 120 compounds referring to the series of both classical and non-classical antifolate analogous agents of formulas **1** [1,2], **2** [3], and **3** [4] (Fig. 1).

The basis of this project was to establish that the quinoxaline ring might replace the pteridine nucleus in methotrexate (MTX) or quinazoline analogues of trimetrexate (TMT) and 5,8-dideazafolic acids on the grounds that the pyrimidine moiety may be substituted with a benzene ring bearing a trifluoromethyl or an amino group, or both, able to increase the affinity for dihydrofolate reductase (DHFR) or thymidylate synthase (TS) enzymes. Moreover, the pyrazine moiety could allocate hindering groups (methyl, phenyl, ethoxycarbonyl) at position 3 and in C-2 a substituted aniline **1**, a substituted benzylamine **2** or *p*-aminobenzoylglutamate **3** to modulate biological interaction. On the other hand, by reversing these substituents on position 6 of the quinoxaline ring, we could obtain quinoxaline isosteric analogues of TMT and 5,8-dideazafolic acids. In these cases we have examined either the methylanilino derivatives (**4**, **5**; Z = NH, *N*-propargyl) [5] or the phenoxymethyl derivatives (**4**, **5**; Z = O) [6] (Fig. 1) and we have found a great number of derivatives endowed with tumor cell-line sensitivity and percent tumor growth inhibition activity between concentrations of $10^{-4}$ and $10^{-5}$ M. Examples of 2-arylquinoxalines supporting their employment in the treatment of tumors were recently described in a patent [7]. Our results of in vitro anticancer activity were very encouraging but no evidence of the suggested antifolate activity had so far been established. Thus

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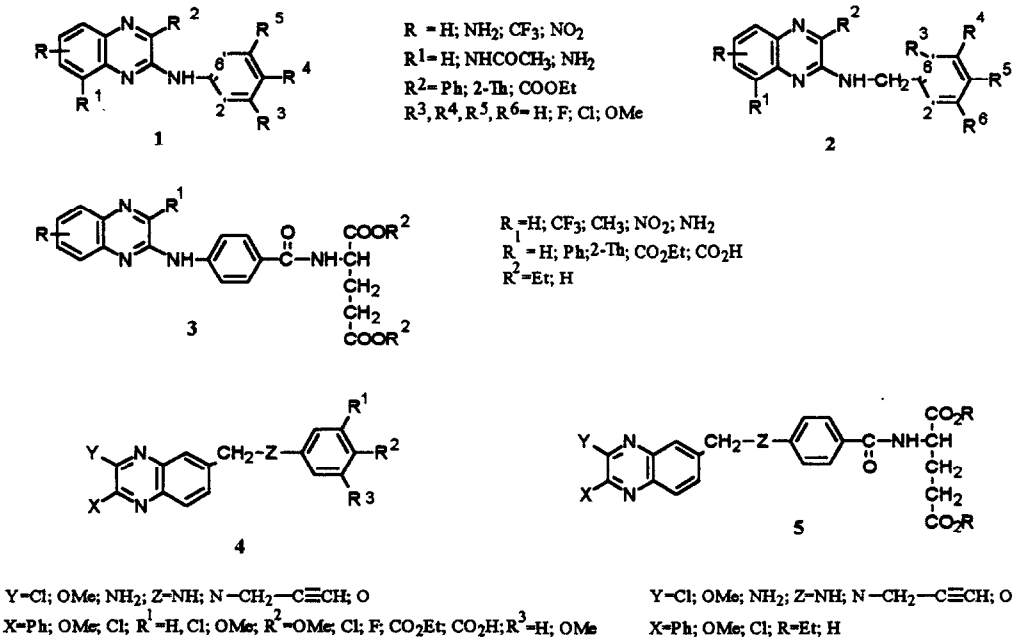
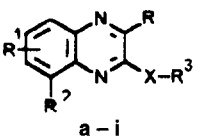



Fig. 1.

we have preliminarily investigated the inhibition of the above-mentioned enzymes from the previously described compounds **a–i** (Table 1) and the results seem now to confirm the grounds for our original assumption.




This prompted us to continue this investigation and in the present paper we have further considered the effect of isosteric replacement of the NH group with oxygen in compounds **6–20** and in some homologues (**21–27**) as well as in those with both *O*-benzoylglutamate (**28–32**) and methyl-*O*-benzoylglutamate (**33–34**) in C-2 depicted in Fig. 2.

## 2. Chemistry

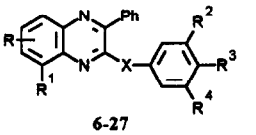
The preparation of the desired compounds was accomplished according to the reactions depicted in Scheme 1.

The chloroquinoxalines **35a–c** and bromomethylquinoxaline **35d** were reacted with the substituted phenols **36** in DMF to give compounds **6–26** in good yields. The acid **27** was obtained in 82% yield on saponification of the ester **26**. On the contrary, the attempts at saponification of the ester **17** produced quinoxalinone **38** (50% yield) accompanied by its

Table 1
Enzymatic inhibition shown by compounds **a–i** against LcTS, HTS and DHFR at the indicated concentration

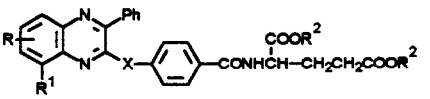
| Comp. [Ref.] | X | R | $R^1$ | $R^2$ | $R^3$ | Conc. [a] (μM) | LcTS | HTS | DHFR (bovine) $IC_{50}$ |
|---|---|---|---|---|---|---|---|---|---|
| **a** [1] | NH | Ph | 6-$CF_3$ | H | 3′,4′,5′-trimethoxyphenyl | 32.8 | NI | NI | NI |
| **b** [1] | NH | Ph | 6-$CF_3$ | $NH_2$ | 3′,4′,5′-trimethoxyphenyl | 10 | NI | NI | NI |
| **c** [3] | NH–$CH_2$ | Ph | 6-$CF_3$ | H | 3′,4′,5′-trimethoxyphenyl | 6.83 | NI | NI | NI |
| **d** [4] | NH | H | 6-$CF_3$ | H | *p*-benzoylglutamate diethyl ester | 10 | NI | NI | NI |
| **e** [4] | NH | H | 6-$CF_3$ | H | *p*-benzoylglutamic acid | 300 | NI | NI | 151 |
| **f** [4] | NH | $CO_2Et$ | H | H | *p*-benzoylglutamate diethyl ester | – | – | – | 114.23 |
| **g** [4] | NH | $CO_2H$ | H | H | *p*-benzoylglutamic acid | – | – | – | 93.8 |
| **h** [4] | NH | $CO_2Et$ | 7-$CF_3$ | H | *p*-benzoylglutamate diethyl ester | – | – | – | 108.4 |
| **i** [4] | NH | $CO_2H$ | 7-$CF_3$ | H | *p*-benzoylglutamic acid | – | – | – | 95.5 |

[a] Maximal concentration used corresponding to the solubility limit.
NI, no inhibition; –, not determined.



6-27

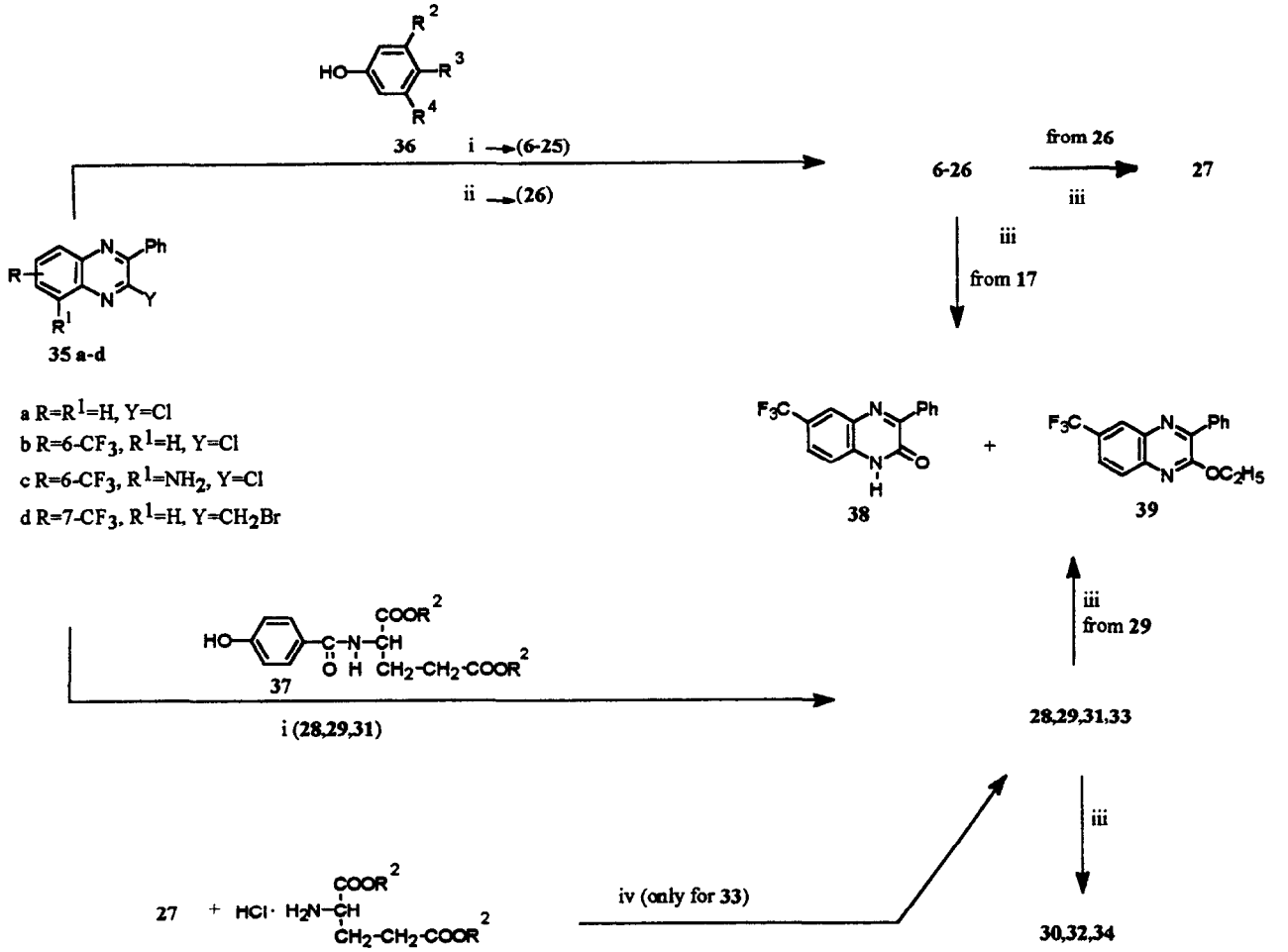
| Compd | X | R | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|---|---|
| 6 | O | H | H | OMe | OMe | OMe |
| 7 | O | H | H | OMe | H | OMe |
| 8 | O | H | H | H | OMe | H |
| 9 | O | H | H | H | CN | H |
| 10 | O | H | H | H | F | H |
| 11 | O | H | H | H | $CO_2Me$ | H |
| 12 | O | 6-$CF_3$ | H | OMe | OMe | OMe |
| 13 | O | 6-$CF_3$ | H | OMe | H | OMe |
| 14 | O | 6-$CF_3$ | H | H | OMe | H |
| 15 | O | 6-$CF_3$ | H | H | CN | H |
| 16 | O | 6-$CF_3$ | H | H | F | H |
| 17 | O | 6-$CF_3$ | H | H | $CO_2Me$ | H |
| 18 | O | 6-$CF_3$ | $NH_2$ | OMe | OMe | OMe |
| 19 | O | 6-$CF_3$ | $NH_2$ | OMe | H | OMe |
| 20 | O | 6-$CF_3$ | $NH_2$ | H | OMe | H |
| 21 | $CH_2O$ | 7-$CF_3$ | H | OMe | OMe | OMe |
| 22 | $CH_2O$ | 7-$CF_3$ | H | OMe | H | OMe |
| 23 | $CH_2O$ | 7-$CF_3$ | H | H | OMe | H |
| 24 | $CH_2O$ | 7-$CF_3$ | H | H | CN | H |
| 25 | $CH_2O$ | 7-$CF_3$ | H | H | F | H |
| 26 | $CH_2O$ | 7-$CF_3$ | H | H | $CO_2Me$ | H |
| 27 | $CH_2O$ | 7-$CF_3$ | H | H | COOH | H |



| Compd | X | R | $R^1$ | $R^2$ |
|---|---|---|---|---|
| 28 | O | H | H | Et |
| 29 | O | 6-$CF_3$ | H | Et |
| 30 | O | H | H | H |
| 31 | O | 6-$CF_3$ | $NH_2$ | Et |
| 32 | O | 6-$CF_3$ | $NH_2$ | H |
| 33 | $CH_2O$ | 7-$CF_3$ | H | Et |
| 34 | $CH_2O$ | 7-$CF_3$ | H | H |

Fig. 2.

2-ethoxy derivative **39** (45% yield), thus indicating a displacement of the substituted phenol moiety. Reaction of the intermediates **35a,b,c** with the purposely prepared *p*-hydroxybenzoylglutamate diethyl ester **37** gave the expected esters **28**, **29** and **31** in good yield (Table 2), whereas to obtain the ester **33** we submitted compound **27** to reaction with diethyl L-glutamate hydrochloride in dimethylformamide and in the presence of diethylcyanophosphonate at room temperature. Saponification of the esters **28**, **31** and **33** gave the corresponding acids **30**, **32** and **34**, while in the case of compound **29** displacement of *p*-hydroxybenzoylglutamate took place, giving rise to the above-mentioned compound **38** (20% yield), whereas compound **39** (74% yield) was dominant. According to the different results obtained during saponification of compounds **26**, **28** and **33** in comparison with those coming from compounds **17** and **29**, it is reasonable to think that the trifluoromethyl group in position 6 plays a certain role in activating the nucleophilic displacement of the phenol derivative at position 2 since in the case of compounds **26**, **28** and **33** this behaviour was not observed. An explanation for this can be supported by an additional elimination mechanism of both the ethoxide and hydroxylic anions at C-2 that rather resembles a cyclic iminoether easily undergoing hydrolysis, whereas an attack on the ester carbonyl is disfavoured (Scheme 2), the influence of the trifluoromethyl group being without effect when a concomitant $NH_2$ substituent was present at position 8 or when the phenol moiety was bound to a methylene bridge. In contrast, as the reaction was




Scheme 1. i: DMF, $CsCO_3$, 70°C, 6 or 13 h; ii: DMF, $CsHCO_3$, 70°C, 2 h; iii: EtOH, 1 M NaOH; iv: DMF, $(EtO)_2POCN$, TEA, r.t., 2 h.

carried out only in aqueous alkaline medium, we recovered the starting material.

The bromomethylquinoxaline **35d** is a new compound and it has been obtained by bromination in acetic acid of the known quinoxaline **40** [8] along with 10% of the dibromo derivative **41** according to Scheme 3.

## 3. Experimental

### 3.1. Chemistry

Melting points are uncorrected and were recorded on a Kofler or an electrothermal melting point apparatus. UV spectra are qualitative and were recorded in nm for solutions in ethanol with a Perkin-Elmer Lambda 5 spectrophotometer. IR spectra are for nujol mulls and were recorded on Perkin-Elmer 781 instruments. $^1H$ NMR spectra were recorded at 200 MHz with a Varian XL-200 instrument using TMS as internal standard. Elemental analyses were performed at the Laboratorio di Microanalisi, Dipartimento di Scienze Farmaceutiche, University of Padua, Italy. The analytical results for C, H, and N were within $\pm 0.4\%$ of the theoretical values.

#### 3.1.1. Intermediates

The chloroquinoxalines **35b,c** have been prepared as previously described in a recent paper [1]. The chloroquinoxaline **35a** [9] and *p*-hydroxybenzoylglutamate **37** [10] were prepared according to the procedures described in the literature cited. The monobromo **35d** and dibromomethylquinoxaline **41** are new compounds and are reported for the first time as described below.

##### 3.1.1.1. 3-Phenyl-7-trifluoromethyl-2-bromomethyl-quinoxaline 35d and 41

Bromine (1.1 g, 6.9 mmol) in acetic acid (4 ml) was added dropwise (10 min), at 20°C, to a vigorously stirred mixture of 3-phenyl-7-trifluoromethyl-2-methylquinoxaline **40**, prepared as described [8] (2 g, 6.9 mmol), and 0.48 g of sodium acetate in acetic acid (40 ml). Then the mixture was heated at 100°C for an additional 30 min under stirring. After cooling the resulting solution was evaporated in vacuo and the red–orange oily residue was taken up with chloroform. An inorganic product was removed by filtration and the mother liquors, dried over anhydrous sodium sulfate, were evaporated to dryness. The solid residue was purified by flash chromatography on a silica gel column eluting with a mixture of ethyl acetate/petrol ether (b.p. 40–60°C) in the ratio 98:2.

Table 2
Melting points, yields analytical and spectroscopic (IR, UV, $^1$H NMR) data of the compounds of Fig. 2

| Comp. | M.p. (°C) [a] | Yield (%) | Analysis for | IR (nujol) $\nu_{max}$ ($cm^{-1}$) | UV (EtOH) $\lambda_{max}$ (nm) | $^1$H NMR, $\delta_H$ Solvent: [A] = $CDCl_3$, [B] = $CDCl_3$:DMSO-$d_6$ (3:1) |
|---|---|---|---|---|---|---|
| **6** | 128–129 (a) | 72 | $C_{23}H_{20}N_2O_4$ | | 344, 243 205 | [A] 8.26–8.10 (3H, m, arom.), 7.85–7.78 (1H, m, arom.), 7.66–7.52 (5H, m, arom.), 6.53 (2H, s, H-2′,6′), 3.89 (3H, s, 4′-OMe), 3.84 (6H, s, 3′,5′-OMe) |
| **7** | 84–85 (a) | 85 | $C_{22}H_{18}N_2O_3$ | | 342, 242, 204 | [A] 8.25–8.05 (3H, m, arom.), 7.85–7.75 (1H, m. arom.), 7.68–7.60 (2H, m, H-2″,6″), 7.56–7.48 (3H, m, H-3″,4″,5″), 6.58–6.36 (3H, m, H-2′,4′,6′), 3.80 (6H, s, 3,5-OMe) |
| **8** | 137–138 (a) | 88 | $C_{21}H_{16}N_2O_2$ | | 340, 240, 204 | [A] 8.27–8.20 (2H, m, H-2″,6″), 8.15–8.08 (1H, m, arom.), 7.78–7.72 (1H, m, arom.), 7.63–7.58 (3H, m, H-3″,4″,5″), 7.55–7.51 (2H, m, arom.), 7.20 (2H, d, $J$ = 9.2 Hz, H-2′,6′), 6.98 (2H, d, $J$ = 9.0 Hz, H-3′,5′), 3.86 (3H, s, 4′-OMe) |
| **9** | 145–147 (a) | 83 | $C_{21}H_{13}N_3O$ | 2220 | 338, 245, 203 | [A] 8.20–8.12 (2H, m, arom.), 7.90–7.55 (4H, m, arom.), 7.72 (2H, d, $J$ = 8.4 Hz, H-2′,6′), 7.56–7.50 (3H, m, H-3″,4″,5″), 7.42 (2H, d, $J$ = 8.8 Hz, H-3′,5′) |
| **10** | 134–135 (a) | 95 | $C_{20}H_{13}FN_2O$ | | 331, 239, 205 | [A] 8.28–8.22 (2H, m, arom.), 8.18–8.10 (1H, m, arom.), 7.80–7.73 (1H, m, arom.), 7.68–7.62 (2H, m, H-2″,6″), 7.62–7.52 (3H, m, H-3″,4″,5″), 7.30–7.10 (4H, m, H-2′,3′,5′,6′) |
| **11** | 119–120 (a) | 67 | $C_{22}H_{16}N_2O_3$ | 1720 | 339, 245, 203 | [A] 8.23–8.12 (3H, m, arom.), 8.15 (2H, d, $J$ = 8.8 Hz, H-2′,6′), 7.78–7.63 (4H, m, arom.), 7.58–7.50 (2H, m, arom.), 7.35 (2H, d, $J$ = 8.6 Hz, H-3′,5′), 3.95 (3H, s, COOMe) |
| **12** | 180–182 (a) | 86 | $C_{14}H_{18}F_3N_2O_4$ | | 340, 242, 205 | [A] 8.43 (1H, d, $J_{5,7}$ = 1.8 Hz, H-5), 8.27–8.20 (2H, m, H-2″,6″), 7.90 (1H, d, $J_{8,7}$ = 8.6 Hz, H-8), 7.82 (1H, dd, $J_{7,8}$ = 8.4 and $J_{7,5}$ = 1.8 Hz, H-7), 7.59–7.51 (3H, m, H-3″,4″,5″), 6.52 (2H, s, H-2′,6′), 3.90 (3H, s, 4′-OMe), 3.86 (6H, s, 3′,5′-OMe) |
| **13** | 92–94 (a) | 97 | $C_{23}H_{17}F_3N_2O_3$ | | 340, 244, 204, 203 | [A] 8.42 (1H, d, $J_{5,7}$ = 1.8 Hz, H-5), 8.26–8.21 (2H, m, H-2″,6″), 7.89 (1H, d, $J_{8,7}$ = 8.6 Hz, H-8), 7.81 (1H, dd, $J_{7,8}$ = 8.6 and $J_{7,5}$ = 1.8 Hz, H-7), 7.57–7.52 (3H, m, H-3″,4″,5″), 6.48–6.42 (3H, m, H-2′,4′,6′), 3.81 (6H, s, 3′,5′-OMe) |
| **14** | 115–116 (a) | 86 | $C_{22}H_{15}F_3N_2O_2$ | | 339, 243, 222, 205 | [A] 8.41 (1H, d, $J_{5,7}$ = 1.8 Hz, H-5), 8.28–8.23 (2H, m, H-7,8), 7.84–7.80 (2H, m, H-2″,6″), 7.58–7.54 (3H, m, H-3″,4″,5″), 7.20 (2H, d, $J$ = 9.0 Hz, H-2′,6′), 6.99 (2H, d, $J$ = 9.0 Hz, H-3′,5′), 3.87 (3H, s, 4′-OMe) |
| **15** | 113–115 (a) | 87 | $C_{22}H_{12}F_3N_3O$ | 2220 | 338, 244, 223, 203 | [A] 8.45 (1H, d, $J_{5,7}$ = 1.8 Hz, H-5), 8.23–8.18 (2H, m, H-2″,6″), 7.85–7.81 (2H, m, H-7,8), 7.80 (2H, d, $J$ = 8.6 Hz, H-2′6′), 7.59–7.56 (3H, m, H-3″,4″,5″), 7.43 (2H, d, $J$ = 8.6 Hz, H-3′,5′) |
| **16** | 121–122 (a) | 83 | $C_{21}H_{12}F_4N_2O$ | | 339, 241, 205 | [A] 8.43 (1H, d, $J_{5,7}$ = 1.8 Hz, H-5), 8.26–8.21 (2H, m, H-2″,6″), 7.88–7.76 (2H, as, H-7,8), 7.58–7.55 (3H, m, H-3″,4″,5″), 7.29–7.12 (4H, m, H-2′,3′,5′,6′) |
| **17** | 123–125 (a) | 73 | $C_{23}H_{15}F_3N_2O_3$ | 1720 | 324, 230, 206 | [A] 8.43 (1H, d, $J_{5,7}$ = 1.8 Hz, H-5), 8.36–8.20 (2H, m, H-2″,6″), 8.17 (2H, d, $J$ = 8.6 Hz, H-2′,6′), 7.85–7.76 (2H, m, H-7,8), 7.60–7.50 (3H, m, H-3″,4″,5″), 7.36 (2H, d, $J$ = 8.6 Hz, H-3′,5′), 3.95 (3H, s, OMe) |

Table 2 (*continued*)

| Comp. | M.p. (°C) [a] | Yield (%) | Analysis for | IR (nujol) $\nu_{max}$ ($cm^{-1}$) | UV (EtOH) $\lambda_{max}$ (nm) | $^1H$ NMR, $\delta_H$ Solvent: [A] = $CDCl_3$, [B] = $CDCl_3$:DMSO-$d_6$ (3:1) |
|---|---|---|---|---|---|---|
| **18** | 198–199 (a) | 56 | $C_{24}H_{20}F_3N_3O_4$ | 3480, 3350 | 348, 288, 206 | [A] 8.27–8.22 (2H, m, H-2″,6″), 7.57–7.54 (3H, m, H-3″,4″,5″), 7.39 (1H, d, $J_{5,7}$ = 1.6 Hz, H-5), 6.96 (1H, d, $J_{7,5}$ = 1.6 Hz, H-7), 6.50 (2H, s, H-2′,6′), 5.19 (2H, s, $NH_2$ [c]), 3.90 (3H, s, 4′-OMe), 3.85 (6H, s, 3′,5′-OMe) |
| **19** | 195–196 (a) | 95 | $C_{23}H_{18}F_3N_3O_3$ | 3500, 3415 | 345, 294, 227, 204 | [A] 8.24–8.21 (2H, m, H-2″,6″), 7.55–7.52 (3H, m, H-3″,4″,5″), 7.41 (1H, d, $J_{5,7}$ = 1.6 Hz, H-5), 6.95 (1H, d, $J_{7,5}$ = 1.6 Hz, H-7), 6.46–6.42 (3H, m, H-2′,4′,6′), 5.17 (2H, s, $NH_2$ [c]), 3.80 (6H, s, 3′,5′-OMe) |
| **20** | 102–103 (a) | 40 | $C_{22}H_{16}F_3N_3O_2$ | 3490, 3390 | 346, 290, 227, 202 | [A] 8.27–8.24 (2H, m, H-2″,6″), 7.55–7.52 (3H, m, H-3″,4″,5″), 7.33 (1H, d, $J_{5,7}$ = 1.6 Hz, H-5), 7.18 (2H, d, $J$ = 9.0 Hz, H-2′,6′), 6.97 (2H, d, $J$ = 9.0 Hz, H-3′,5′), 6.29 (1H, d, $J_{7,5}$ = 1.6 Hz, H-7), 5.15 (2H, s, 8-$NH_2$ [c]), 3.85 (3H, s, 4′-OMe) |
| **21** | 119–120 (a) | 41 | $C_{25}H_{21}F_3N_2O_4$ | | 326, 238, 206 | [A] 8.50 (1H, d, $J_{8,6}$ = 2.0 Hz, H-8), 8.31 (1H, d, $J_{5,6}$ = 8.8 Hz, H-5), 7.99 (1H, dd, $J_{6,5}$ = 8.8 and $J_{6,8}$ = 2.0 Hz, H-6), 7.86 (2H, m, H-2″, 6″), 7.60–7.55 (3H, m, H-3″,4″,5″), 6.19 (2H, s, H-2′,6′), 5.33 (2H, s, $CH_2$), 3.80 (6H, s, 3′,5′-OMe), 3.78 (3H, s, 4′-OMe) |
| **22** | 67–68 (a) | 40 | $C_{24}H_{19}F_3N_2O_3$ | | 325, 237, 205 | [A] 8.52 (1H, d, $J_{8,6}$ = 2.0 Hz, H-8), 8.30 (1H, d, $J_{5,6}$ = 8.6 Hz, H-5), 7.98 (1H, dd, $J_{6,5}$ = 8.8 and $J_{6,8}$ = 2.0 Hz, H-6), 7.80–7.75 (2H, m, H-2″,6″), 7.54–7.51 (3H, m, H-3″,4″,5″), 6.20–6.11 (3H, m, H-2′,4′,6′), 5.30 (2H, s, $CH_2$), 3.75 (6H, s, 3′,5′-OMe) |
| **23** | 94–95 (b) | 55 | $C_{23}H_{17}F_3N_2O_2$ | | 325, 237, 204 | [A] 8.52 (1H, d, $J_{8,6}$ = 2.0 Hz, H-8), 8.30 (1H, d, $J_{5,6}$ = 9.0 Hz, H-5), 7.98 (1H, dd, $J_{6,5}$ = 9.0 and $J_{6,8}$ = 2.0 Hz, H-6), 7.82–7.77 (2H, m, H-2″,6″), 7.54–7.50 (3H, m, H-3″,4″,5″), 6.84 (4H, q, $J$ = 9.4 Hz, H-2′,3′,4′,5′), 5.29 (2H, s, $CH_2$), 3.77 (3H, s, OMe) |
| **24** | 108–109 (b) | 42 | $C_{23}H_{14}F_3N_3O_3$ | 2216 | 324, 241, 204 | [A] 8.49 (1H, d, $J_{8,6}$ = 2.0 Hz, H-8), 8.31 (1H, d, $J_{5,6}$ = 8.8 Hz, H-5), 8.00 (1H, dd, $J_{6,5}$ = 8.8 and $J_{6,8}$ = 2.0 Hz, H-6), 7.78–7.72 (2H, m, H-2″,6″), 7.58 (2H, d, $J$ = 8.8 Hz, H-2′,6′), 7.56–7.50 (3H, m, H-3″,4″,5″), 6.99 (2H, d, $J$ = 8.8 Hz, H-3′,5′), 7.41 (2H, s, $CH_2$) |
| **25** | 61–63 (a) | 47 | $C_{22}H_{14}F_4N_2O$ | | 325, 237, 206 | [A] 8.51 (1H, d, $J_{8,6}$ = 1.8 Hz, H-8), 8.33 (1H, d, $J_{5,6}$ = 9.0 Hz, H-5), 7.99 (1H, dd, $J_{6,5}$ = 9.0 and $J_{6,8}$ = 1.8 Hz, H-6), 7.85–7.63 (2H, m, H-2″,6″), 7.62–7.50 (3H, m, H-3″,4″,5″), 7.03–6.79 (4H, m, H-2′,3′,5′,6′), 5.31 (2H, s, $CH_2$) |
| **26** | 96–96 (a) | 62 | $C_{24}H_{17}F_3N_2O_3$ | 1720 | 327, 239, 205 | [A] 8.50 (1H, d, $J_{8,6}$ = 1.8 Hz, H-8), 8.30 (1H, d, $J$ = 5.6 Hz, H-5), 7.98 (3H, d, $J$ = 9.0 Hz, H-2′,6′ + H-6 obscured), 7.78–7.73 (2H, m, H-2″,6″), 7.53–7.50 (3H, m, H-3″,4″,5″), 6.95 (2H, d, $J$ = 9.0 Hz, H-3′,5′), 5.40 (2H, s, $CH_2$), 3.88 (3H, s, COOMe) |
| **27** | 207–208 (a) | 73 | $C_{23}H_{15}F_3N_2O_3$ | 1690 | 325, 239, 204 | [A] 8.51 (1H, d, $J_{8,6}$ = 1.8 Hz, H-8), 8.32 (1H, d, $J_{5,6}$ = 8.4 Hz, H-5), 8.05 (2H, d, $J$ = 8.4 Hz, H-2′,6′), 8.01 (1H, dd [b], $J$ = 8.4 and 1.8 Hz, H-6), 7.84–7.70 (2H, m, H-2″,6″), 7.60–7.48 (3H, m, H-3″,4″,5″), 6.98 (2H, d, $J$ = 8.4 Hz, H-3′,5′), 5.43 (2H, s, $CH_2$) |

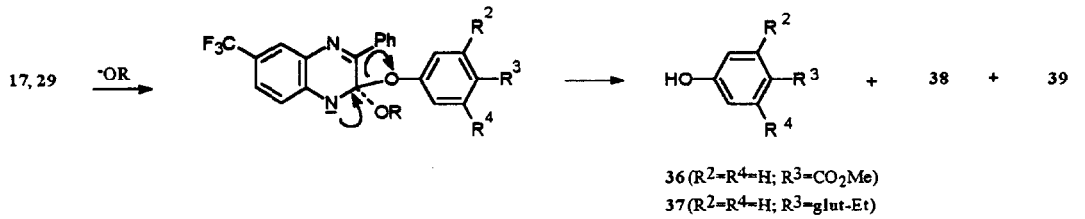
Table 2 (*continued*)

| Comp. | M.p. (°C) [a] | Yield (%) | Analysis for | IR (nujol) $\nu_{max}$ ($cm^{-1}$) | UV (EtOH) $\lambda_{max}$ (nm) | $^1H$ NMR, $\delta_H$ Solvent: [A] = $CDCl_3$, [B] = $CDCl_3$:DMSO-$d_6$ (3:1) |
|---|---|---|---|---|---|---|
| **28** | 148–149 (a) | 77 | $C_{30}H_{29}N_3O_6$ | 3220, 1755, 1730, 1640 | 341, 247, 204 | [A] 8.23–8.10 (2H, m, arom.), 7.94 (2H, d, $J$ = 8.6 Hz, H-2′,6′), 7.77–7.60 (4H, m, H-2″,6″ + 2H arom.), 7.60–7.50 (3H, m, H-3″,4″,5″), 7.36 (2H, d, $J$ = 8.6 Hz, H-3′,5′), 7.14 (1H, d, $J$ = 7.2 Hz, N*H*CH [c]), 4.88–4.76 (1H, m, $CH_2CH$–NH), 4.26 (2H, q, $CH_2CH_3$), 4.13 (2H, q, $CH_2CH_3$), 2.63–2.06 (4H, m, $CH_2CH_2CH$), 1.32 (3H, t, $CH_3CH_2$), 1.24 (3H, t, $CH_3CH_2$) |
| **29** | 174–175 (a) | 77 | $C_{31}H_{28}F_3N_3O_6$ | 3300, 1740, 1640 | 339, 244, 222, 204 | [A] 8.44 (1H, d, $J_{8,6}$ = 1.8 Hz, H-8), 8.27–8.21 (2H, m, H-2″,6″), 7.97 (2H, d, $J$ = 8.8 Hz, H-2′,6′), 7.83 (2H, ad, H-7,8), 7.59–7.55 (3H, m, H-3″,4″,5″), 7.37 (2H, d, $J$ = 8.8 Hz, H-3′,5′), 7.17 (1H, d, $J$ = 7.4 Hz, N*H*CH [c]), 4.94–4.77 (1H, m, NHC*H*$CH_2$), 4.27 (2H, q, $CH_2CH_3$), 4.14 (2H, d, $CH_2CH_3$), 2.60–2.10 (4H, m, $CH_2CH_2CH$), 1.33 (3H, t, $CH_3CH_2$), 1.25 (3H, t, $CH_3CH_2$) |
| **30** | 203–204 (a) | 82 | $C_{26}H_{21}N_3O_6$ | 3300, 1720, 1700, 1635 | 340, 246, 204 | [B] 8.27–8.10 (4H, m, arom.), 8.03 (2H, d, $J$ = 8.4 Hz, H-2′,6′), 7.76–7.63 (3H, m, arom.), 7.63–7.52 (2H, m, arom.), 7.35 (2H, d, $J$ = 8.4 Hz, H-3′,5′), 4.78–4.60 (1H, m, NHC*H*$CH_2$), 4.35 (3H, brs, 2·COOH [c]), 2.65–2.06 (4H, m, $CH_2CH_2CH$) |
| **31** | 145–146 (c) | 57 | $C_{31}H_{29}F_3N_4O_6$ | 3480, 3380, 3280, 1740 | 344, 295, 242, 203 | [A] 8.26–8.20 (2H, m, 2″,6″), 7.95 (2H, d, $J$ = 8.8 Hz, H-2′,6′), 7.57–7.53 (3H, m, H-3″,4″,5″), 7.35 (2H, d, $J$ = 8.8 Hz, H-3′,5′), 7.33 (1H, d obscured, H-7), 7.13 (1H, d, $J$ = 7.4 Hz, NH [c]), 6.96 (1H, d, $J_{7,5}$ = 1.8 Hz, H-7), 5.21 (2H, s, $NH_2$ [c]), 4.84 (1H, m, NHC*H*$CH_2$), 4.26 (2H, q, $CH_2CH_3$), 4.14 (2H, q, $CH_2CH_3$), 2.60–2.10 (4H, m, $CH_2CH_2$), 1.32 (3H, t, $CH_3CH_2$), 1.25 (3H, t, $CH_3CH_2$) |
| **32** | 225–227 (d) | 83 | $C_{27}H_{21}F_3N_4O_6$ | 3400, 3300, 1740, 1640 | 345, 295, 243, 203 | [B] 8.31–8.24 (3H, m, H-2″,6″ + NH [c]), 8.04 (2H, d, $J$ = 8.4 Hz, H-2′,6′), 7.60–7.55 (3H, m, H-3″,4″,5″), 7.34 (2H, d, $J$ = 8.4 Hz, H-3′,5′), 7.12 (1H, s, H-5), 7.02 (1H, s, H-7), 5.80 (2H, brs, $NH_2$ [c]), 4.73–4.60 (1H, m, CH), 4.05 (2H, brs, 2·COOH [c]), 2.60–2.10 (4H, m, $CH_2CH_2$) |
| **33** | 89–90 (b) | 81 | $C_{32}H_{30}F_3N_3O_6$ | 3290, 1730, 1640 | 325, 239, 204 | [A] 8.50 (1H, d, $J_{8,6}$ = 2.0 Hz, H-8), 8.30 (1H, d, $J_{5,6}$ = 8.6 Hz, H-5), 7.99 (1H, dd, $J_{6,5}$ = 8.6 and $J_{6,8}$ = 2.0 Hz, H-6), 7.86–7.75 (2H, m, H-2″,6″), 7.77 (2H, d, $J$ = 8.4 Hz, H-2′,6′), 7.60–7.52 (3H, m, H-3″,4″,5″), 7.00 (1H, d [b], CHN*H* [c]), 6.96 (2H, d, $J$ = 8.4 Hz, H-3′,5′), 5.40 (2H, s, $CH_2$), 4.87–4.75 (1H, m, $CH_2CH$NH), 4.23 (2H, q, $CH_2CH_3$), 4.14 (2H, q, $CH_2CH_3$), 2.61–2.06 (4H, m, $CH_2CH_2CH$), 4.30 (3H, t, $CH_3CH_2$), 1.26 (3H, t, $CH_3CH_2$) |
| **34** | 146–150 (d) | 83 | $C_{28}H_{22}F_3N_3O_6$ | 3310, 1720, 1690, 1640 | 325, 239, 205 | [B] 8.49 (1H, d, $J_{8,6}$ = 2.0 Hz, H-8), 8.32 (1H, d, $J_{5,6}$ = 8.8 Hz, H-5), 8.01 (H, dd, $J_{6,5}$ = 8.8 and $J_{6,8}$ = 2.0 Hz, H-6), 7.83 (2H, d, $J$ = 8.8 Hz, H-2′,6′), 7.83–7.74 (2H, m, H-2″,6″), 7.65 (1H, d, $J$ = 6.8 Hz, N*H*CH [c]), 7.57–7.50 (3H, m, H-3″,4″,5″), 6.95 (2H, d, $J$ = 8.8 Hz, H-3′,5′), 5.41 (2H, s, $CH_2$), 4.74–4.60 (1H, m, $CH_2CH$NH), 2.62–2.05 (4H, m, $CH_2CH_2$) |

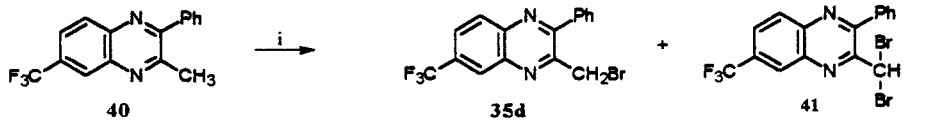
[a] Purification procedure: (a) crystallized from ethanol; (b) crystallized from methanol; (c) crystallized from a mixture of methanol and water; (d) crystallized from acetic acid.

[b] Partially obscured by other resonances.

[c] Exchanges with $H_2O$.




Scheme 2.




Scheme 3. i: $Br_2$, $CH_3COOH$, $CH_3COONa$, 30 min, 100°C.

The most mobile fraction gave on evaporation compound **41** (0.3 g, 10% yield), m.p. 121–122°C.

*Anal.* ($C_{16}H_9Br_2F_3N_2$) C, H, N; UV: 332, 243, 205. $^1$H NMR ($CDCl_3$): $\delta$ 8.62 (1H, d, $J_{8,7}$ = 1.8 Hz, H-8), 8.28 (1H, d, $J_{5,6}$ = 9.0 Hz, H-5), 8.10 (1H, dd, $J_{6,5}$ = 9.0 and $J_{6,8}$ = 1.8 Hz, H-6), 7.76–7.68 (2H, m, H-2',6'), 7.68–7.58 (3H, m, H-3',4',5'), 6.97 (1H, s, $CHBr_2$).

The successive fractions gave an evaporation compound **35d** (1.65 g, 65% yield), m.p. 131–133°C from ethanol.

*Anal.* ($C_{16}H_{10}BrF_3N_2$) C, H, N; UV: 328, 241, 206. $^1$H NMR ($CDCl_3$): $\delta$ 8.46 (1H, d, $J_{8,7}$ = 1.8 Hz, H-8), 8.26 (1H, d, $J_{5,6}$ = 8.8 Hz, H-5), 7.96 (1H, dd, $J_{6,5}$ = 8.8 and $J_{6,8}$ = 1.8 Hz, H-6), 7.82–7.77 (2H, m, H-2',6'), 7.63–7.54 (3H, m, H-3',4',5'), 4.77 (2H, s, $CH_2Br$).

### *3.1.2. General procedure for preparation of the 2-phenoxy and 2-phenoxymethylquinoxalines 6–26*

A mixture of equimolar amounts (1 mmol) of 2-chloroquinoxalines **35a–c** or 2-bromoethylquinoxaline **35d** and the corresponding substituted phenol (Fig. 2) in anhydrous DMF (15 ml) in the presence of one mole equivalent of caesium carbonate was stirred at 70°C for 13 h (**6–20**) or 6 h (**21–25**). In the case of compound **26**, caesium hydrogencarbonate was used and the mixture heated at 70°C for 2 h. After cooling, water was added to complete precipitation of solids (**6–18, 20, 23**) which were collected and washed with water and eventually dried. Compounds **19, 21, 22** and **24–26** separated as oils and were extracted with ether or chloroform. The organic phase, dried over anhydrous sodium sulfate and evaporated in vacuo, gave solid compounds. Purification methods, yields, melting points, analytical and spectroscopic data are reported in Table 2.

### *3.1.3. 4-(3-Phenyl-7-trifluoromethylquinoxalin-2-yl) methoxybenzoic acid 27*

A suspension of the ester **26** (0.44 g, 1 mmol) in a mixture of ethanol (12 ml) and 1 M NaOH aqueous solution (6 ml), was stirred at 70°C for 4 h. The clear solution which formed after evaporation of the solvent was diluted with water and made acidic with 2 M HCl aqueous solution. A cream coloured solid precipitated which was first washed with water, then recrystallized from ethanol. Yields, melting points, analytical and spectroscopic data are reported in Table 2.

### *3.1.4. Attempts at saponification of the esters 17 and 29, formation of 2-ethoxy-3-phenyl-6-trifluoromethyl-quinoxaline 39 and 3-phenyl-6-trifluoromethyl-quinoxalinyl-1H-2-one 38*

(i) A suspension of the ester **17** (0.6 g, 1.4 mmol) in a mixture of EtOH (30 ml), water (20 ml) and 1 M NaOH aqueous solution (6 ml) was heated at 70–75°C under stirring for 24 h. On cooling, a white product was filtered off and thoroughly washed with water to give compound **39** (0.2 g, 45% yield), m.p. 114–116°C.

*Anal.* ($C_{17}H_{13}F_3N_2O$) C, H, N; UV: 338, 288, 238, 212, 205. $^1$H NMR ($CDCl_3$): $\delta$ 8.37 (1H, s, H-5), 8.17–8.12 (2H, m, H-2',6') 7.93 (1H, d, $J$ = 8.6 Hz, H-8), 7.82 (1H, dd, $J$ = 8.6 and 1.8 Hz, H-7), 7.54–7.50 (3H, m, H-3',4',5'), 4.66 (2H, q, $CH_2$–$CH_3$), 1.52 (3H, t, $CH_2$–$CH_3$).

The mother liquor, on acidification with 1 M HCl aqueous solution, formed a precipitate which after filtration gave compound **38** (0.29 g, 50% yield), identical with an authentic specimen previously described [10].

(ii) A suspension of the ester **29** (0.25 g) was treated with a mixture of EtOH (4 ml) and 1 M NaOH aqueous solution (3 ml) at room temperature for 10 h. The work-up of the mixture gave compounds **39** (0.1 g, 74% yield) and **38** (0.03 g, 20% yield), identical with the above-described samples.

### *3.1.5. General procedure for preparation of compounds 28, 29, 31 and 33*

(i) A mixture of equimolar amounts (1 mmol) of **35a,b,c** and diethyl 4-hydroxy-benzoyl-L-glutamate, dissolved in anhydrous DMF (10–15 ml) and in the presence of one mole equivalent of caesium carbonate, was stirred at room temperature for 13 h. Then it was diluted with water and the precipitates which formed were collected and washed with water. The white products (**28, 29, 31**) were further purified by recrystallization from ethanol. Yields, melting points, analytical and spectroscopic data are reported in Table 2.

(ii) Diethyl cyanophosphonate (0.125 g, 0.77 mmol) in DMF (3 ml) and TEA (0.15 g, 1.48 mmol) in DMF (3 ml)

were added under a continuous stream of nitrogen to a mixture of **27** (0.3 g, 0.71 mmol) and diethyl-L-glutamate hydrochloride (0.185 g, 0.77 mmol) in DMF (12 ml). The mixture was stirred for 4 h and then poured onto a mixture (3:1 ratio) of ethyl acetate and benzene (40 ml). The organic phase was washed, in order, with water (50 ml), saturated sodium carbonate solution (60 ml), water (50 ml), and saturated sodium chloride solution (60 ml) and eventually dried over anhydrous sodium sulfate. On evaporation, compound **33** was obtained as a solid and purified as indicated in Table 2, which also reports yields, melting points, analytical and spectroscopic data.

### *3.1.6. General procedure for preparation of the acids 30, 32, 34*

A suspension of ester (**28, 31, 33**) (0.8 mmol) in a mixture of ethanol (8 ml) and 1 M NaOH aqueous solution (5 ml) was stirred at room temperature for 5 h. On evaporation of the solvent, the mixture was taken up with water and made acidic with 2 M HCl aqueous solution. The pale yellow solids formed (**30**, **32**, **34**) were collected and washed with water then recrystallized by the solvent indicated in Table 2, which also reports yields, melting points, analytical and spectroscopic data.

## *3.2. Pharmacology*

Evaluation of anticancer and anti-HIV activity was performed on 24 out of the 29 compounds referring to structures **9**, **10**, **12–31**, **33**, **34** of Fig. 2 and Scheme 1 at the National Cancer Institute (NCI), Bethesda, Md, USA, following the well-known [11] in vitro disease-oriented antitumor screening program against a panel of 60 human tumor cell lines and the anti-HIV drug testing system [12]. No compounds exhibited anti-HIV activity. The anticancer activity of each compound is deduced from dose–response curves and is presented in three different tables according to the data provided by NCI. In Table 3 the response parameters $GI_{50}$, TGI and $LC_{50}$ refer to the concentration of the agents in the assay that produced 50% growth inhibition, total growth inhibition, and 50% cytotoxicity, respectively, and are expressed as mean graph midpoints. In Table 4 we report the activities of those compounds which showed a percent growth inhibition greater than 40% on subpanel cell lines at a concentration of $10^{-4}$ M. In Table 5 we report the activities of those compounds which showed a percent growth inhibition greater than 40% on subpanel cell lines at $10^{-5}$ M.

### *3.2.1. Results of the in vitro pharmacological anticancer assays*

The data of in vitro anticancer activity reported in Table 3 established that the average sensitivity of all cell lines towards the tested agent, represented as mean graph midpoints, falls in the concentration range $10^{-5.53}$–$10^{-4}$ M. A good correlation exists between the values of $-\log GI_{50}$, $-\log TG$ and $-\log LC_{50}$, although the most active compound (**18**) was not the most cytotoxic. A survey of Table 4 better indicates the highest activities recorded on all the panel cell lines for the compounds **12**, **15**, **16**, **18**, **19**, **20**, **27**, **28**, **29**, **31** and **33**, while a few compounds (**10**, **14**, **21**, **25**) exhibited a moderate range of both cell sensitivity and percent tumor growth inhibition, the remaining compounds (**9**, **13**, **17**, **22**, **23**, **24**, **26**, **34**) exhibiting only random cell sensitivity and moderate or no tumor growth inhibition activity. Among the derivatives of the first group compound, **18** maintained high values of percent tumor growth inhibition on all cell lines at $10^{-5}$ M, followed to a lesser extent by compound **31** > **28** ≥ **29**. A few random cell line sensitivities were recorded for the other compounds at this concentration. Interestingly, a large number of derivatives (**10**, **13**, **14**, **16**, **18**, **19**, **21**, **22**, **23**, **24**, **25**, **27**, **31**, **33**) exhibited very significant percent tumor growth inhibition activities between $10^{-8}$ and $10^{-6}$ M (Table 6). Among these compounds **18** and **31** were the most active.

Table 3
$-\log_{10}GI_{50}$, $-\log_{10}TGI$, $-\log_{10}LC_{50}$ mean graph midpoints [a] of the in vitro inhibitory activity tests for compounds **9**, **10**, **12–31**, **33** and **34** and against human tumor cell lines [b]

| Comp. | $-\log_{10}GI_{50}$ | $-\log_{10}TGI$ | $-\log_{10}LC_{50}$ |
|---|---|---|---|
| **9** | 4.00 | 4.00 | 4.00 |
| **10** | 4.06 | 4.00 | 4.00 |
| **12** | 4.59 | 4.15 | 4.03 |
| **13** | 4.02 | 4.00 | 4.00 |
| **14** | 4.20 | 4.00 | 4.00 |
| **15** | 4.74 | 4.40 | 4.12 |
| **16** | 4.21 | 4.01 | 4.00 |
| **17** | 4.04 | 4.00 | 4.00 |
| **18** | 5.53 | 4.44 | 4.04 |
| **19** | 4.47 | 4.07 | 4.00 |
| **20** | 4.58 | 4.18 | 4.04 |
| **21** | 4.15 | 4.00 | 4.00 |
| **22** | 4.07 | 4.00 | 4.00 |
| **23** | 4.03 | 4.00 | 4.00 |
| **24** | 4.09 | 4.00 | 4.00 |
| **25** | 4.28 | 4.02 | 4.00 |
| **26** | 4.01 | 4.00 | 4.00 |
| **27** | 4.36 | 4.03 | 4.01 |
| **28** | 4.68 | 4.25 | 4.08 |
| **29** | 4.59 | 4.09 | 4.01 |
| **30** | 4.00 | 4.00 | 4.00 |
| **31** | 5.01 | 4.18 | 4.01 |
| **33** | 4.26 | 4.04 | 4.01 |
| **34** | 4.01 | 4.00 | 4.00 |

[a] Mean graph midpoints (MG-MIDs), the average sensitivity of all cell lines toward the test agent.
[b] From NCI.

## *3.3. Enzymology*

### *3.3.1. Materials and methods*

Thymidylate synthase from *Lactobacillus casei* and human thymidylate synthase were purposefully prepared as reported below. Dihydrofolate reductase (DHFR) from bovine liver and rat liver was available from SIGMA as a suspension in 3.6 M ammonium sulfate solution at pH 8 and a suspension

Table 4
Percent tumor growth inhibition recorded on subpanel cell lines at $10^{-4}$ M of compounds **9**, **10**, **12–29**, **31**, **33** and **34**

| Panel/Cell-Lines | 9 | 10 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 31 | 33 | 34 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Leukemia** | | | | | | | | | | | | | | | | | | | | | | | |
| CCRF-CEM | - | - | 75 | - | 50 | 151 | nt | - | nt | nt | 64 | 50 | - | - | 57 | 105 | - | 72 | - | - | - | 60 | - |
| HL-60(TB) | - | 80 | 81 | - | 81 | 146 | 54 | 43 | 95 | 128 | 60 | 50 | 43 | - | 90 | 109 | 49 | 95 | - | - | 57 | 46 | - |
| K-562 | - | 46 | 71 | - | 53 | 131 | nt | 61 | 130 | 137 | - | 55 | - | 40 | 56 | 118 | 56 | 84 | - | - | nt | 60 | - |
| MOLT-4 | - | - | nt | - | 56 | 153 | 81 | 55 | 90 | 105 | 76 | 62 | - | - | 53 | 119 | 42 | nt | 44 | - | - | 74 | 42 |
| RPMI-8226 | - | 55 | 66 | 59 | 90 | nt | nt | 63 | 98 | 108 | 61 | 64 | - | - | 46 | 76 | - | nt | nt | nt | - | 64 | nt |
| SR | - | 66 | 66 | - | 83 | 191 | 116 | 51 | 87 | 107 | 86 | 52 | 45 | nt | 56 | 82 | 73 | 94 | 93 | - | nt | 72 | 70 |
| **Non Small Cell Lung Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| A549/ATCC | nt | nt | 133 | - | nt | 167 | 81 | - | 80 | nt | nt | 68 | - | - | - | 45 | - | 86 | 66 | 67 | 91 | 46 | - |
| EKVX | - | - | 83 | - | - | 169 | - | 63 | 73 | 90 | 176 | 51 | 89 | - | 58 | 56 | 49 | 66 | 69 | 106 | 84 | 40 | - |
| HOP-62 | - | 52 | 129 | - | - | 189 | 99 | - | 93 | 40 | 145 | nt | - | - | - | - | - | 56 | 137 | 104 | nt | nt | - |
| HOP-92 | - | 56 | 134 | - | 67 | 183 | nt | - | 73 | - | 84 | 49 | - | - | - | 97 | - | 92 | 187 | 56 | 60 | 54 | - |
| NCI-H226 | - | - | 83 | - | - | nt | nt | - | 58 | 69 | 147 | - | 51 | - | nt | nt | - | 53 | 189 | 176 | 81 | 79 | 50 |
| NCI-23 | - | 66 | 87 | - | 94 | 186 | 55 | - | 92 | 51 | 71 | 52 | - | - | - | 40 | - | 86 | 143 | 100 | 139 | 53 | - |
| NCI-H322M | - | - | nt | nt | - | 177 | - | nt | 65 | 48 | 76 | 60 | nt | - | - | - | - | 61 | 56 | 58 | 70 | 43 | - |
| NCI-H460 | - | - | 70 | - | 42 | 200 | 58 | 55 | 99 | 98 | 128 | 45 | - | - | - | 47 | 50 | 91 | - | 59 | 74 | 61 | - |
| NCI-H522 | - | 56 | 94 | - | - | 178 | 48 | 51 | 115 | nt | nt | nt | - | nt | 70 | 86 | - | 60 | 136 | 94 | 119 | - | - |
| **Colon Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| COLO 205 | - | - | 92 | - | - | 139 | - | - | 93 | 119 | 198 | - | - | - | - | 44 | - | 175 | - | 49 | - | 196 | - |
| HCC-2998 | - | - | 72 | - | 93 | 188 | 158 | 49 | 82 | 100 | 74 | - | 40 | - | - | 50 | - | 83 | - | - | 60 | nt | - |
| HCT-116 | - | - | 154 | 45 | 43 | 200 | 54 | 45 | 87 | 94 | 83 | 51 | 43 | - | - | 50 | 48 | 86 | 180 | 68 | 124 | 63 | - |
| HCT-15 | - | - | - | - | 41 | 200 | 40 | - | 89 | 126 | 78 | - | 49 | - | nt | 66 | - | 88 | - | - | 47 | 58 | - |
| HT29 | - | - | 84 | 56 | 62 | 173 | 89 | 45 | 157 | 88 | 198 | - | 59 | - | 63 | 120 | 42 | 90 | 200 | 110 | 85 | 49 | - |
| KM12 | - | - | 58 | - | - | 142 | - | - | 92 | 127 | 164 | 48 | - | - | - | 44 | - | 98 | 58 | 65 | 73 | 80 | - |
| SW-620 | - | - | nt | - | - | 200 | nt | - | 70 | 81 | 67 | - | 40 | - | - | 43 | - | 71 | - | - | nt | 42 | - |
| **SNC Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| SF-268 | - | - | 102 | - | - | 112 | - | - | 89 | 56 | 119 | - | - | - | - | 42 | - | 79 | 134 | 107 | 118 | 73 | - |
| SF-295 | - | 50 | 159 | - | - | 132 | 87 | - | 96 | 69 | 156 | 69 | - | - | - | - | - | 97 | 147 | 100 | 111 | 79 | - |
| SF-539 | nt | nt | 122 | - | nt | 105 | 92 | - | 136 | 50 | 69 | 70 | - | - | - | 41 | - | 115 | 200 | 80 | 95 | - | - |
| SNB-19 | - | 45 | nt | nt | - | 170 | 54 | nt | 129 | 51 | 98 | 51 | - | - | - | 53 | - | 62 | 122 | 99 | 116 | - | - |
| SNB-75 | nt | nt | 180 | - | nt | 169 | 89 | - | 103 | 66 | 154 | 50 | - | - | - | 40 | - | 100 | 193 | 168 | 123 | 116 | - |
| U251 | - | 64 | 180 | - | - | 200 | nt | - | 66 | 71 | 92 | 73 | - | - | - | 40 | - | 78 | 103 | 72 | nt | - | - |
| **Melanoma** | | | | | | | | | | | | | | | | | | | | | | | |
| LOX IMVI | - | - | 88 | - | - | 104 | 50 | - | 80 | 148 | 128 | 44 | - | - | - | - | - | 79 | 75 | 67 | 43 | 49 | - |
| MALME-3M | - | - | 53 | - | - | 142 | 99 | - | 145 | nt | nt | 40 | - | - | 43 | 63 | nt | nt | 160 | 127 | 104 | 55 | - |
| M14 | nt | nt | nt | nt | nt | 200 | 74 | nt | 88 | 70 | 139 | 68 | - | - | - | 52 | - | 56 | 69 | 51 | - | 54 | - |
| SK-MEL-2 | - | - | nt | nt | - | 178 | 60 | nt | 124 | 129 | 190 | - | - | - | - | - | - | 77 | 100 | 144 | 143 | 134 | - |
| SK-MEL-28 | - | - | 68 | - | - | 200 | 72 | - | 60 | 59 | 53 | 60 | - | - | - | - | - | 62 | 80 | 47 | nt | - | - |
| SK-MEL-5 | - | - | 135 | - | - | 145 | 62 | - | 114 | 74 | 78 | 85 | nt | - | 42 | 77 | 69 | 94 | nt | nt | 77 | 139 | nt |
| UACC-257 | - | - | 65 | - | - | 133 | 69 | - | 110 | 55 | 76 | - | 50 | - | - | 88 | 41 | 56 | - | 62 | nt | 59 | - |
| UACC-62 | - | - | 54 | - | - | 89 | 80 | - | 145 | 46 | 90 | 57 | - | - | - | 49 | - | 90 | 78 | 84 | 79 | 66 | - |
| **Ovarian Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| IGROV1 | - | - | 67 | - | - | 156 | - | - | 146 | 99 | 136 | 49 | 67 | nt | - | - | - | 68 | 95 | 85 | 89 | 54 | - |
| OVCAR-3 | - | - | 101 | - | - | 156 | - | - | 135 | nt | nt | 41 | - | - | - | - | - | 79 | 140 | 98 | 146 | 54 | - |
| OVCAR-4 | - | 80 | 153 | - | - | 182 | 68 | 47 | nt | 54 | 42 | 51 | - | 40 | - | 51 | - | nt | 106 | 91 | 80 | 146 | - |
| OVCAR-5 | - | - | - | - | - | 200 | 40 | - | 51 | nt | nt | - | - | - | - | 42 | - | 45 | 80 | - | 71 | - | - |
| OVCAR-8 | nt | nt | 72 | - | nt | 164 | 43 | - | 150 | 68 | 97 | 58 | - | - | - | - | - | 77 | 200 | 153 | 90 | 50 | - |
| SK-OV-3 | - | - | 155 | - | - | 186 | 53 | - | 77 | 71 | 135 | 50 | - | - | nt | nt | nt | nt | 151 | 142 | 135 | - | - |
| **Renal Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| 786-0 | - | 55 | 175 | - | - | 200 | 63 | - | 88 | 65 | 128 | 51 | - | - | - | - | - | 50 | 200 | 124 | 174 | 49 | - |
| A498 | - | - | 98 | - | - | 189 | nt | - | 104 | - | 143 | 40 | nt | 51 | - | - | - | 48 | 98 | 96 | - | - | - |
| ACHN | - | - | 136 | - | - | 200 | 78 | 61 | 118 | 100 | 93 | 43 | - | - | - | 42 | - | 125 | 121 | 87 | 112 | 51 | - |
| CAKI-1 | - | - | 200 | - | - | 160 | 118 | - | 151 | 99 | 159 | 46 | - | - | - | - | - | 189 | 143 | 171 | 151 | 59 | 67 |
| RXF 393 | - | 90 | 128 | - | 62 | 112 | 62 | 56 | 112 | 85 | 191 | 117 | - | - | nt | nt | - | 89 | 123 | 104 | 168 | 53 | - |
| SN12C | - | - | 88 | - | - | 165 | 46 | - | 78 | nt | nt | nt | - | - | - | 42 | - | 65 | nt | 79 | 55 | 46 | - |
| TK-10 | - | - | 122 | - | - | 200 | 71 | - | 58 | 60 | 81 | 56 | - | - | - | 100 | - | 60 | 140 | 65 | 72 | nt | - |
| UO-31 | - | - | 41 | - | 69 | 139 | 74 | - | 66 | 42 | 64 | 43 | - | - | 41 | 44 | - | 185 | 82 | 84 | 65 | 78 | - |
| **Prostate Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| PC-3 | - | - | 84 | - | - | 200 | 41 | - | 119 | 85 | 124 | 50 | - | - | - | - | - | 70 | 50 | 70 | 107 | 67 | - |
| DU-145 | - | - | - | - | - | 183 | - | - | 65 | nt | nt | 52 | - | - | - | - | - | 63 | 78 | - | 56 | 53 | - |
| **Breast Cancer** | | | | | | | | | | | | | | | | | | | | | | | |
| MCF7 | - | - | 90 | 48 | 56 | 153 | 99 | 72 | 93 | 100 | 200 | 58 | - | - | 50 | 60 | - | 84 | nt | 41 | 73 | 197 | - |
| MCF7/ADR-RES | nt | nt | 41 | - | nt | 163 | - | - | 87 | 62 | nt | 55 | - | - | - | - | - | 78 | nt | nt | 98 | 61 | nt |
| MDA-MB-231/ATCC | 41 | - | 76 | - | 41 | 153 | - | - | 77 | 101 | 100 | 42 | 52 | 64 | 55 | - | - | 67 | nt | 127 | 76 | - | - |
| HS 578T | 49 | 52 | 97 | - | - | 152 | 100 | - | nt | 55 | 144 | 64 | 61 | - | 40 | 56 | - | 77 | nt | nt | 114 | 87 | nt |
| MDA-MB-435 | - | - | 70 | - | 40 | 183 | 58 | - | 82 | 112 | 70 | 43 | - | - | - | 70 | - | 68 | 81 | - | nt | 54 | - |
| BT-549 | - | 51 | 128 | - | 75 | 97 | - | - | 124 | 44 | 74 | 46 | 44 | - | - | 99 | - | 57 | nt | nt | 104 | 63 | nt |
| T-47D | nt | nt | 61 | 65 | nt | 157 | 50 | 60 | 83 | 125 | 162 | 77 | 56 | 100 | 46 | 59 | 44 | 70 | 119 | 85 | 71 | 86 | - |
| MDA-N | - | - | 70 | 47 | - | 130 | 50 | - | 115 | nt | nt | - | - | - | - | 49 | - | 77 | 113 | 49 | 57 | 73 | - |

–, below 40% growth inhibition; nt, not tested at this molar concentration.

Table 5
Percent tumor growth inhibition recorded on subpanel cell lines at $10^{-5}$ M of compounds **10**, **12–14**, **18–30**, **31**, **33** and **34**

| Panel/Cell-Lines | 10 | 12 | 13 | 14 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 33 | 34 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Leukemia** | | | | | | | | | | | | | | | | | | | | |
| CCRF-CEM | - | - | - | - | 56 | 44 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| HL-60(TB) | 45 | - | - | - | 85 | - | - | - | - | - | 67 | - | - | - | - | - | - | - | - | - |
| K-562 | - | - | - | - | 112 | 64 | - | - | - | - | - | 79 | - | - | - | - | - | nt | nt | - |
| MOLT-4 | - | - | - | - | 76 | 51 | - | 51 | - | - | 40 | 62 | - | nt | - | - | - | - | - | - |
| RPMI-8226 | - | - | - | 57 | 71 | - | - | 47 | - | - | 46 | - | - | nt | nt | nt | nt | 42 | - | nt |
| SR | - | - | - | 46 | 86 | 47 | - | - | - | nt | - | - | - | nt | 51 | - | 48 | nt | 44 | 50 |
| **Non Small Cell Lung Cancer** | | | | | | | | | | | | | | | | | | | | |
| A549/ATCC | nt | - | - | nt | 62 | nt | nt | - | - | - | - | - | - | - | - | - | - | 90 | - | - |
| HOP-62 | - | 47 | - | - | 64 | - | - | nt | - | - | - | - | - | - | - | 66 | - | nt | nt | - |
| HOP-92 | - | - | - | - | - | - | - | 40 | - | - | - | - | - | - | 71 | 46 | - | - | - | - |
| NCI-H226 | - | - | - | - | - | - | 42 | - | - | - | nt | nt | - | - | 52 | 60 | - | 62 | 46 | - |
| NCI-23 | - | - | - | 113 | 65 | - | - | - | - | - | - | - | - | - | 41 | - | - | 54 | - | - |
| NCI-H322M | - | nt | nt | - | 51 | - | - | - | nt | - | - | - | - | - | - | - | - | - | - | - |
| NCI-H460 | - | - | - | - | 77 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| NCI-H522 | - | - | - | - | 75 | nt | nt | nt | - | nt | 53 | 46 | - | - | 45 | 51 | - | 63 | - | - |
| **Colon Cancer** | | | | | | | | | | | | | | | | | | | | |
| COLO 205 | - | 41 | - | - | 61 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| HCC-2998 | - | - | | - | 54 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| HCT-116 | - | 47 | - | - | 66 | - | - | - | - | - | - | - | - | - | 49 | - | - | 111 | - | - |
| HCT-15 | - | - | - | - | 75 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| HT29 | - | - | - | - | 146 | 41 | 45 | - | 42 | - | 55 | - | - | - | 122 | 70 | - | 59 | - | - |
| KM12 | - | - | - | - | 84 | - | - | - | - | - | - | - | - | - | - | 51 | - | 44 | - | - |
| SW-620 | - | - | - | - | 55 | - | - | - | - | - | - | - | - | - | - | - | - | nt | | - |
| **SNC Cancer** | | | | | | | | | | | | | | | | | | | | |
| SF-268 | - | - | - | - | 71 | - | - | - | - | - | - | - | - | - | 56 | 55 | - | 56 | - | - |
| SF-29 | - | 51 | - | - | 84 | - | - | - | - | - | - | - | - | - | - | - | - | 75 | - | - |
| SF-539 | nt | - | - | nt | 68 | - | - | - | - | - | - | - | - | - | 141 | 88 | - | 153 | - | - |
| SNB-19 | - | nt | nt | - | 73 | - | - | - | - | - | - | - | - | - | - | 47 | - | - | - | - |
| SNB-75 | nt | 52 | - | nt | 69 | - | - | - | - | - | - | - | - | - | 57 | 42 | - | 52 | 60 | - |
| U251 | - | - | - | - | 50 | - | - | - | - | - | - | - | - | - | 54 | 48 | - | nt | - | - |
| **Melanoma** | | | | | | | | | | | | | | | | | | | | |
| LOX IMVI | - | - | - | - | 64 | - | - | - | - | - | - | - | - | - | - | - | - | 42 | - | - |
| MALME-3M | - | - | - | - | 116 | nt | nt | - | - | - | - | - | nt | nt | 43 | - | - | 49 | - | - |
| M14 | nt | nt | nt | nt | 53 | - | - | - | - | - | - | - | - | - | - | - | - | 44 | - | - |
| SK-MEL-2 | - | nt | nt | - | 84 | - | - | - | - | - | - | - | - | - | - | - | - | 103 | - | - |
| SK-MEL-5 | - | - | - | - | 69 | - | - | - | nt | - | - | - | - | - | nt | nt | nt | 47 | - | nt |
| UACC-257 | - | - | - | - | 64 | - | - | - | - | - | - | 57 | - | - | - | - | - | 55 | - | - |
| UACC-62 | - | 45 | - | - | 77 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Ovarian Cancer** | | | | | | | | | | | | | | | | | | | | |
| IGROV1 | - | - | - | - | 110 | - | - | - | 72 | nt | - | - | - | - | - | - | - | - | - | - |
| OVCAR-3 | - | - | - | - | 88 | nt | nt | - | - | - | - | - | - | - | - | - | - | 102 | - | - |
| OVCAR-4 | - | - | - | - | nt | - | - | - | - | - | - | - | - | nt | 65 | 58 | - | 57 | - | - |
| OVCAR-5 | - | - | - | - | - | nt | nt | - | - | - | - | - | - | - | - | - | - | 93 | - | - |
| OVCAR-8 | nt | - | - | nt | nt | - | - | - | - | - | - | - | - | - | 86 | 42 | - | 78 | - | - |
| SK-OV-3 | - | - | 40 | - | 56 | - | - | - | - | - | nt | nt | nt | nt | - | - | - | 59 | - | - |
| **Renal Cancer** | | | | | | | | | | | | | | | | | | | | |
| 786-0 | - | - | - | - | 65 | - | - | - | - | - | - | - | - | - | 108 | 71 | - | 134 | - | - |
| A498 | - | - | - | - | 54 | - | - | - | nt | - | - | - | - | - | - | - | - | - | - | - |
| ACHN | - | 44 | - | - | 66 | - | - | - | - | - | - | - | - | - | 61 | 68 | - | 74 | - | - |
| CAKI-1 | - | 69 | - | - | 77 | - | - | - | - | - | - | - | - | 64 | - | 82 | - | 113 | - | - |
| RXF 393 | - | 47 | - | - | 54 | - | 40 | - | - | - | nt | nt | - | - | - | - | - | 82 | - | - |
| SN12C | - | - | 54 | - | 48 | nt | nt | nt | - | - | - | - | - | - | nt | - | - | - | - | - |
| TK-10 | - | - | - | - | - | - | - | - | - | - | - | 82 | - | - | 58 | 51 | - | 58 | nt | - |
| UO-31 | - | - | - | 57 | 44 | - | - | - | | - | - | - | - | - | - | - | - | - | - | - |
| **Prostate Cancer** | | | | | | | | | | | | | | | | | | | | |
| PC-3 | - | - | - | - | 82 | - | - | - | - | - | - | - | - | - | - | - | - | 74 | - | - |
| DU-145 | - | - | - | - | 46 | nt | nt | - | - | - | - | - | - | - | - | - | - | 41 | - | - |
| **Breast Cancer** | | | | | | | | | | | | | | | | | | | | |
| MCF7 | - | 53 | - | - | 77 | 52 | 40 | - | - | - | - | - | - | - | nt | - | - | - | 61 | - |
| MCF7/ADR-RES | nt | - | - | nt | 70 | nt | nt | - | - | - | - | - | - | - | nt | - | nt | 85 | - | nt |
| MDA-MB-231/ATCC | - | - | - | - | 44 | - | - | - | - | - | - | - | - | - | nt | 63 | - | - | - | - |
| HS 578T | - | - | - | - | 68 | - | - | - | - | - | - | - | - | - | nt | nt | nt | 62 | - | nt |
| MDA-MB-435 | - | - | - | - | 75 | 46 | - | - | - | - | - | - | - | - | - | - | - | nt | - | - |
| BT-549 | - | - | - | 113 | 52 | - | - | 42 | 45 | - | - | - | - | - | nt | nt | nt | - | - | nt |
| T-47D | nt | - | - | nt | 53 | 106 | 53 | 48 | - | 43 | - | - | - | - | 52 | - | - | - | - | - |
| MDA-N | - | - | - | - | 106 | nt | nt | - | - | - | - | - | - | - | - | - | - | - | - | - |

–, below 40% growth inhibition; nt, not tested at this molar concentration.

Table 6
Comparison of the inhibitory activity of compounds on some cell lines at the most diluted concentrations

| Cell line | Comp. | Percent tumor growth inhibition at indicated molar concentration (M) | | |
|---|---|---|---|---|
| | | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
| **Leukemia** | | | | |
| HL-60(TB) | **10** | 27 | 24 | 20 |
| HL-60(TB) | **18** | 50 | 14 | nt |
| K-562 | **19** | 39 | 21 | |
| K-562 | **24** | | 33 | 54 |
| K-562 | **25** | 89 | 31 | |
| MOLT-4 | **21** | 30 | 26 | 23 |
| SR | **14** | 29 | 9 | 18 |
| SR | **18** | 66 | 44 | 16 |
| SR | **28** | 36 | 24 | 32 |
| **Non small cell lung cnacer** | | | | |
| A549/ATCC | **31** | 21 | 30 | 29 |
| HOP-62 | **26** | 25 | 24 | 24 |
| HOP-62 | **27** | 28 | | 22 |
| NCI H226 | **31** | 47 | | |
| NCI H226 | **33** | 47 | 26 | |
| NCI-H23 | **14** | 47 | | |
| NCI-H522 | **25** | 32 | | |
| **Colon cancer** | | | | |
| HCC-2998 | **16** | 36 | 28 | 28 |
| HCT-116 | **18** | 30 | | |
| HCT-116 | **31** | 55 | | |
| HT-29 | **18** | 50 | | |
| HT-29 | **31** | 61 | 48 | |
| KM-12 | **18** | 40 | 25 | 21 |
| **CNS** | | | | |
| SF-295 | **14** | | | 37 |
| SF-539 | **31** | 38 | | |
| SNB-75 | **18** | 56 | 27 | |
| SNB-75 | **27** | 27 | 33 | |
| SNB-75 | **33** | 35 | | |
| **Melanoma** | | | | |
| MALME-3M | **18** | 80 | 15 | |
| **Ovarian cancer** | | | | |
| IGROV1 | **18** | 67 | 22 | |
| IGROV1 | **22** | 53 | 24 | 16 |
| OVCAR-5 | **31** | 35 | 26 | |
| **Renal cancer** | | | | |
| A498 | **23** | 35 | 37 | |
| RXF-393 | **31** | 60 | 35 | |
| SN12C | **13** | 37 | 17 | |
| TK-10 | **25** | 56 | | |
| UO-31 | **14** | 41 | | |
| **Prostate cancer** | **18** | 40 | | |
| **Breast cancer** | | | | |
| NCI/ADR-RES | **31** | 81 | 61 | |
| MCF7/ADR-RES | **24** | | 38 | |
| MDA-MB-435 | **18** | 58 | | |
| BT-549 | **10** | | 25 | 45 |
| BT-549 | **14** | 77 | 16 | 18 |
| MDA-N | **18** | 78 | | |

in glycerol, 5%, 0.7 M ammonium sulfate, pH 6.5, respectively. The enzymatic assays have been run on a UV–Vis spectrophotometer, Beckman DU 640, equipped with a thermostated circulating bath, HAAKEF3C.

### 3.3.2. Experimental

Plasmids that express *L. casei* TS (TS, EC 2.1.1.45) in the Thy-*Escherichia coli* strain X2913 have been described [13]. The enzyme was purified by a column chromatography method using phosphocellulose (P11, Biorad) and hydroxyapatite (HAP, Biorad) resin, with phosphate buffer as eluant [14]. Human thymidylate synthase (HTS) was purified as reported using affinity column chromatography [15]. The enzyme preparations were >95% homogeneous, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The LcTS purified enzyme was stored at −80°C in 10 mM phosphate buffer, pH 7.0, 0.1 mM EDTA until use. HTS was used immediately after purification. The activity of TS was determined by steady-state kinetic analysis, spectrophotometrically, by following the increasing absorbance at 340 nm due to the oxidation reaction of N5,N10-methylene tetrahydrofolate to dihydrofolate at the 5,6 bond [16]. Assays were performed at 20°C in the standard assay buffer which contained 50 mM *N*-tri(hydroxymethyl-2-aminoethane) (TES) at pH 7.4, 25 mM $MgCl_2$, 6.5 mM formaldehyde, 1 mM EDTA and 75 mM 2-mercaptoethanol. 1 ml of reaction mixture is formed by the standard TES buffer, pH 7.4, dUMP 120 mM, 6-(*R,S*)-1-$CH_2CH_4$-folate 180 mM, enzyme 0.07 mM.

The stock solutions of the inhibitors were prepared in DMSO with a concentration of about 2 mM and kept at −20°C. Control reactions were run in order to measure the effect of DMSO on enzyme activity. DMSO never exceeded 5% in the enzyme assay mixture [17].

TS inhibition studies were run under the general conditions of standard TS assays with the exception of the folate concentration which was kept at 100 mM. The assay conditions are those of the standard TS assay. DHFR (DHFR, EC 1.5.1.3) catalyses the NADPH dependent reduction of dihydrofolate ($H_2$folate) to tetrahydrofolate ($H_4$folate). The assays were run spectrophotometrically by measuring the decrease in absorbance at 340 nm upon NADPH reduction at 25°C. One unit of enzyme is defined as one nmol of $H_2$folate reduced per minute.

The reaction mixture was formed by 50 mM TES, pH 7.0, EDTA 1 mM, 75 mM 1-mercaptoethanol, 100 mM NADPH, 58.03 mM $H_2$folate [18].

All the experiments were repeated at least three times and the standard errors from non-linear least-square fits of the experimental data are less than 20% for all the values.

$IC_{50}$ values (inhibitor concentration that inhibits the enzyme activity at 50% of the control) were measured when the solubility of the compound allowed the experimental measures. In all the other cases only the percentage of inhibition at a fixed concentration was measured and calculated values of $IC_{50}$ ($IC_{50c}$, where c is for calculated) were determined from the dependence of the initial rates on the inhibitor concentration using the equation $V_i/V_0 = (1 - C_i/IC_{50})$, where $V_i$ and $V_0$ are the initial rates in the presence and absence of inhibitor, and $C_i$ is the concentration of inhibitor [19].

Table 7
Inhibitory activity of compounds **12**, **15**, **18**, **19**, **27**, **29**, **31** and **33** against LcTS, HTS and DHFR at the indicated concentration (μM)

| Comp. | Bovine liver DHFR | | Rat liver DHFR | | LcTS | HTS |
|---|---|---|---|---|---|---|
| | *I*% (at fixed conc.) | $IC_{50}$ [a] (μM) | *I*% (at fixed conc.) | $IC_{50}$ [a] (μM) | | |
| **12** | NI (28.95) | | | | NI (28.95) | NI (28.95) |
| **15** | | 46.2 ($IC_{50}$) | | 70.5 ($IC_{50}$) | NI (200) | NI (200) |
| **18** | | 88.9 ($IC_{50}$) | 25.7 (28.3) | 81.8 ($IC_{50c}$) | NI (100) | NI (100) |
| **19** | 30.4 (14.25) | 22 ($IC_{50c}$) | 33 (14.25) | 28.84 ($IC_{50c}$) | NI (14.25) | NI (14.25) |
| **27** | | 186 ($IC_{50}$) | 39.4 (67) | 103.1 ($IC_{50c}$) | NI (200) | NI (200) |
| **29** | 7.9 (11.1) | 129.7 ($IC_{50}$) | | | NI (11.1) | NI (11.1) |
| **31** | | 18.45 ($IC_{50}$) | 18.7 (7.8) | 36.3 ($IC_{50c}$) | NI (31.2) | NI (31.2) |
| **33** | 33.6 (9.78) | 38.6 ($IC_{50c}$) | 17.2 (9.78) | 42.7 ($IC_{50c}$) | NI (9.78) | NI (9.78) |

NI, no inhibition at the indicated concentrations in parentheses corresponding to the solubility limit.
[a] Experimental $IC_{50}$ or $IC_{50c}$ (c for calculated) as reported in Section 3.

*3.3.3. Results of inhibitory enzymatic assays*

The data of Table 1 refer to compounds previously described in other papers as cited. From these it appears evident that no type of quinoxaline tested was active against LcTS and HTS, whereas in some cases (**e–i**), water solubility permitting a certain inhibitory activity against bovine DHFR was detected. The structure of the compounds represented in Table 1 belongs to both the non-classical (**a–c**) and classical (**d–i**) folate analogue series. It is important to note that in this test only classical folate analogues showed some inhibitory activity.

Among the novel compounds described here, only **12**, **15**, **18**, **19**, **27**, **29**, **31** and **33**, corresponding to those endowed with the highest percent growth inhibition activity, have been assayed against both bovine and rat liver DHFR enzymes, which are considered a good model for mammalian enzymes [20], as well as against LcTS and HTS (Table 7). Comparison of the data of Table 1 with those of Table 7 clearly showed that compounds with an ether bridge (**12**, **15**, **18**, **19**, **27**, **29**, **31**, **33**) exhibited the highest inhibitory activity against DHFR from bovine liver with respect to compounds **a–i** listed in Table 1. No compound of this type was active against both LcTS and HTS. Interestingly, compounds exhibiting some inhibitory activity (Table 7) against bovine DHFR showed more or less comparable inhibitory activity against rat liver DHFR, indicating that no selectivity can be observed between the two species. Owing to the poor water solubility of most compounds, only the percent inhibition or calculated $IC_{50}$ ($IC_{50c}$) could be determined. $IC_{50}$ was measured only for compounds **15**, **19**, **27**, **29**, **31** and **33** (Table 7).

## 4. Discussion and conclusions

All the classical and non-classical folate analogues are active anticancer agents, but they did not show TS inhibitory activity, indicating that TS is not the target for these compounds inside the cell. In compounds of the previous series (**e–i**) bearing an NH bridge with a glutamate moiety in the side chain a moderate inhibitory activity was observed against bovine DHFR, where compound **f** ($IC_{50} = 114.23$ μM) was as active as its trifluoromethyl analogue **h** ($IC_{50} = 108.4$ μM). The results obtained from either in vitro anticancer or DHFR evaluation for compounds with an ether bridge seem to indicate an increase of inhibitory activity against DHFR, suggesting that this enzyme can be the main target inside the cell; the only exception was compound **12** which was inactive at 28.95 μM against DHFR from bovine liver. Compounds **15**, **18** **19**, **27**, **29**, **31** and **33** were endowed either with high percent tumor growth inhibition activity at $10^{-4}$ M (Table 4) or with moderate to high inhibitory activity against DHFR enzymes (Table 7). Compound **18** was the most active in the anticancer test, while compound **31** was the most active against DHFR in the enzymatic assay.

Structure–activity relationships show that the most active compound **18** in the anticancer test is a non-classical folate analogue, where the presence of an amino group in position 8 increases the activity, expressed as mean graph midpoints (Table 3), in comparison with compound **12**. This behaviour is much more marked in the DHFR assay since compound **12** is inactive, while **18** is active ($IC_{50} = 88.9$ μM). It is interesting to note that compound **29** exhibited poor inhibition activity against DHFR from bovine liver ($IC_{50c} = 129.7$ μM) in comparison with compound **31** ($IC_{50} = 18.4$ μM), which instead differs from it by the presence of the 8-amino group, as in compounds **18** and **12** in the same assay were the most active of the classical series, thus suggesting that this group may increase affinity for the enzyme. The deletion of one methoxy group from **18** in the benzene ring increases its inhibitory activity ($IC_{50} = 88.9$ μM) almost three times against **19** ($IC_{50c} = 33$ μM), while this compound in the anticancer test was less active. Comparing compounds **15** and **12**, the cyano group on the benzene moiety gives an $IC_{50}$ of 46.2 μM, against the inactivity of **12**. Considering the classical folate analogue series the introduction of the glutamate moiety in compound **33** compared to **27** increases the inhibitory activity against bovine liver DHFR ($IC_{50c} = 38.6$ μM and $IC_{50} = 186$ μM, respectively) about four times. The same trend can be observed against rat liver DHFR ( $IC_{50c} = 47.2$ and 103 μM, respectively), while their behaviour in the anti-

cancer test show that their activities are of the same order of magnitude (Tables 3 and 4). Compound **31** has both the modifications of the amino group at position 8 and the glutamate moiety and showed the highest inhibitory activity against DHFR, which was in line with the high percent tumor growth inhibition recorded. At this stage it seems reasonable to conclude that DHFR enzymes are the target of the compounds described and that isosteric replacement of the NH with the O bridge is profitable for both anticancer and anti-DHFR activity.

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