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Quinoxaline chemistry. Part 15. 4-[2-Quinoxalylmethylenimino]-benzoylglutamates and -benzoates, 4-[2-quinoxalylmethyl-*N*-methylamino]-benzoylglutamates as analogues of classical antifolate agents. Synthesis, elucidation of structures and in vitro evaluation of antifolate and anticancer activities

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Abstract

We report on an extension of our previous discovery of in vitro anticancer activity of trifluoromethylquinoxalines as analogues of classical and non-classical antifolic methotrexate and trimetrexate. In this case a small number of Schiff bases were obtained from the reaction of 2-bromethyl-3-*R*-6(7)trifluoromethylquinoxaline and ethyl *p*-aminobenzoylglutamate, ethyl *p*-aminobenzoate, *p*-toluidine instead of the expected 4-[2-quinoxalyl]methyl-*N*-methylanilino derivatives, which in turn formed with *N*-methylanilino derivatives. The reaction mechanism has been put forward. Structure elucidation of both Schiff bases and *N*-methylanilino analogues was achieved by a combination of ^{1}H and ^{13}C NMR spectra and hetcor experiments. Compounds **3a**, **3b**, **3c**, **8**, **11**, **12**, **13**, **Ie** were tested in antifolic enzyme assay [*Lactobacillus casei* (LcTS), *Leishmania major* (LmTs), human Thymidylate synthase (hTs), human TS, human dihydrofolate reductase (hDHFR)] while compounds **3a**, **3b**, **3c** were tested for anticancer activity. These results seem to indicate that the Schiff bases are somewhat active either as anticancer or as folate inhibitors, while compound **Ie** was selectively active against hDHFR with an inhibition constant (Ki) of 200 nM with a specificity of about 1000-folds with respect to hTS.

Keywords: Antifolate agents; Anticancer agents; Enzyme inhibition; Dihydrofolate reductase and tymidylate synthase inhibitors; ^{1}H and ^{13}C NMR trifluoromethylquinoxalines

1. Introduction

In the early stage of our research on the quinoxalines as classical analogues of antifolic methotrexate we had considered the synthesis of some compounds closely related to the model. Thus, we had designed compounds of formula **Ia**–**e** in order to investigate them in vitro anticancer activity (Fig. 1).

The reasons for the presence of both 3-phenyl and 6(7)CF_3 ring substitution had been previously supported by the biological data of the series of quinoxaline derivatives meantime published [1–14]. These compounds were neither reported nor discussed because their formation could not be achieved according to a satisfactory procedure that we previously used in similar cases. In fact, in contrast with the expected behaviour, from the reaction of a heteroarylmethyl halide (**1a**–**d**) with an aniline derivative (**2**) no methylamino derivative was obtained whereas the Schiff bases (**3a**–**d**) were instead the products (Fig. 2). Nor the attempts of reduction of the (formed) imino derivatives **3a**–**d**, using different reagents, were successful. However, when bromomethylquinoxaline (**1c**) was reacted with **9** the

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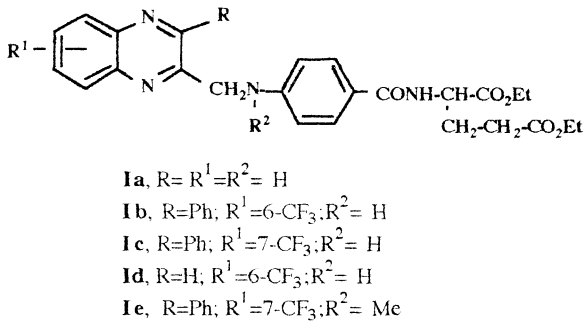

Ia, R= R^1=R^2= H
Ib, R=Ph; R^1=6-CF_3;R^2= H
Ic, R=Ph; R^1=7-CF_3;R^2= H
Id, R=H; R^1=6-CF_3;R^2= H
Ie, R=Ph; R^1=7-CF_3;R^2= Me

Fig. 1. List of compounds to be obtained according to Fig. 2.

desired compound (**Ie**) was obtained. Thus the compounds (**Ie**, **3a**–**c**, **7**, **11**, **12**, **13**) of Figs. 2 and 3 were subjected to pharmacological screening for either anticancer activity or enzymatic assay against different species of Thymidylate synthase (TS) such as *Lactobacillus casei* (LcTS), *Leishmania major* (LmTS), human (hTS) and human dihydrofolate reductase (hDHFR); the results are here presented. In the chemistry section we discuss the particular cases of formation of the products described and elucidation of their structures.

2. Chemistry

The reaction of the bromomethylquinoxalines (**1a**–**d**) with the ethyl *p*-amminobenzoylglutamate (**2**) carried out in dry dimethylacetamide (DMA) gave unexpectedly the imino derivatives (**3a**–**d**) while using the *N*-methyl benzoylglutamate (**9**) from **1c** we obtained **Ie** as expected (Fig. 2, route a). Elucidation of the structure of **3a**–**d**

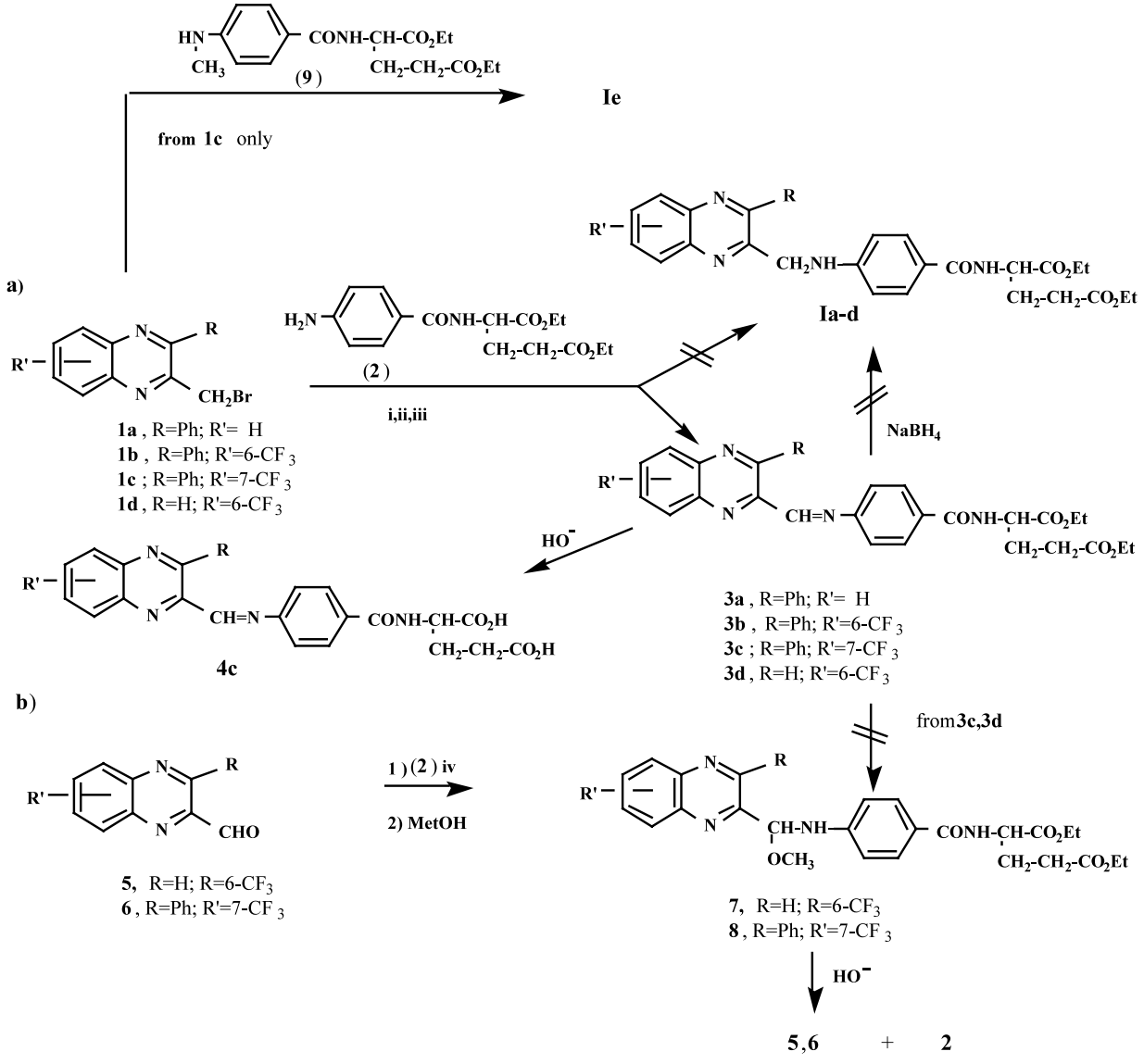

Fig. 2. (i) Dimethylacetamide (DMA), room temperature, 72 h; (ii) DMA, 50 °C, 24 h; (iii) DMA, TEA, 100 °C, 10 h; (iv) melting the mixture at 130 °C, 40 min in vacuo.

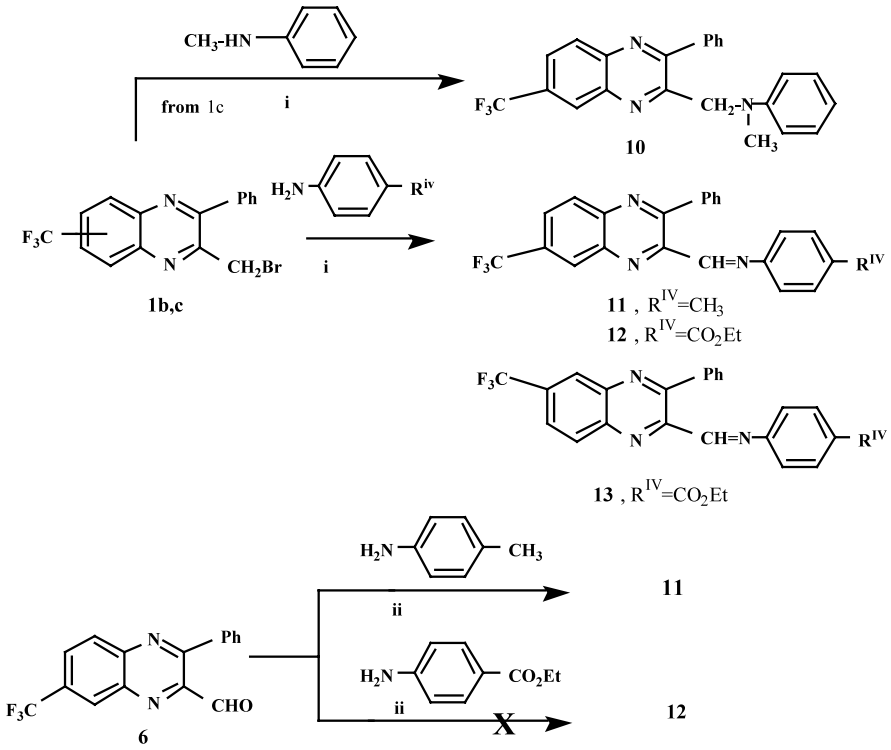

Fig. 3. (i) DMA, 72 h, room temperature; (ii) melting of the mixture at 100–110 °C.

came from an extensive study of their ^{1}H and ^{13}C NMR spectra and hetcor experiments that well correlated the 9-H with C-9. In fact, 9-H resonates as singlet at rather low field (δ 9.66–9.71 range) whereas the ^{13}C chemical shift of C-9 falls at δ l23.85 as doublet that is upper field in comparison with the literature data for aldimines [15].

At this point it seemed reasonable that a similar result might be obtained from the reaction of the aldehydes (**5**, **6**) with **2** in order to obtain compounds **3c** and **3d**. None of these compounds could be isolated, and instead in their place we obtained compounds **7** and **8**. Elucidation of the structure of **7** and **8** came from the elemental analyses and the examination of their ^{1}H and ^{13}C NMR spectra and from the hetcor experiments that will be discussed later. From the ester **3c** by alkaline hydrolysis we obtained the acid **4c** while from the aminals (**7**, **8**) we recovered the starting aldehydes (**5**, **6**) and the ethyl *p*-aminobenzoylglutamate (**2**) (Fig. 2).

These results were quite surprising since the heteroarylmethylhalides in general give with the primary amines the expected secondary amines as reported in the literature [16]. This prompted us to investigate if this type of reaction took place with similar reagents. The confirmed formation of the anil derivatives was then achieved from the results of the reaction of the bromomethylquinoxalines (**1b**, **1c**) with both ethyl *p*-aminobenzoate and *p*-toluidine, which possess similar reactivity as **2** (Fig. 3).

As it is shown in Fig. 3 the bromomethylquinoxaline (**1c**) with *N*-methylaniline gave the expected compound **10** as in the case of **Ie**, whereas with *p*-toluidine and the ethyl *p*-aminobenzoate gave the anils **11** and **12**. In similar manner from **1b** with the ethyl *p*-aminobenzoate we obtained the anil **13**. The reaction of the aldehyde (**6**) with *p*-toluidine yielded the anil (**11**) identical to the above cited authentic specimen. Conversely, on melting the ethyl *p*-aminobenzoate and the aldehyde (**6**) we presume that compound **12** might be formed but in no way could it be isolated from the reaction mixture as such. The ^{1}H NMR spectra of **7** and **8** run in $CDCl_3$ showed that the resonance of C-9 proton moved upfield (δ 6.25) because of shielding effect of the methoxy group and both signals (CH and OMe) disappeared when the spectrum of the compounds was re-run on the residue of its melting point, the other resonances remaining unchanged. ^{13}C NMR spectrum and DEPT experiments show that the signal of C-9 moved upfield (δ 81.00) in comparison with that observed in the Schiff bases (**3a**–**d**). In the light of these results we conclude that formation of the anil derivatives may come according to the mechanism depicted in Fig. 4 that is supported by many examples of tautomerism of the methylene group in position 2 or 3 of quinoxaline ring, well documented in the literature [17]. As a consequence, the secondary amine formed during the condensation may exist in equilibrium with the dihydroquinoxaline (enamine)

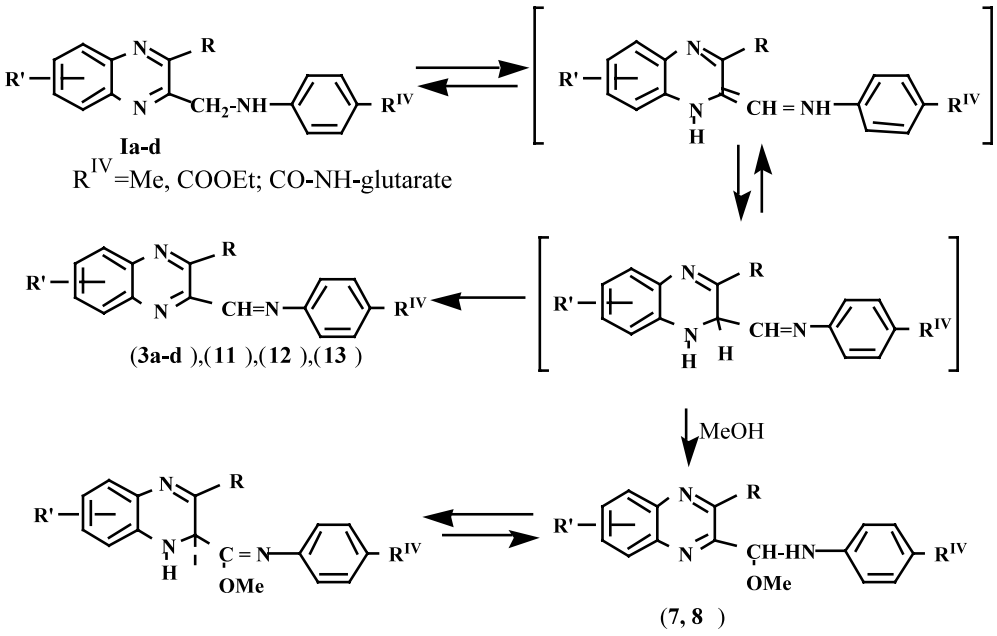

Fig. 4. Reaction mechanism for compounds of Figs. 2 and 3.

form easily undergoing aromatisation to quinoxaline of the intermediate anil. At the present stage we cannot explain the different result when the aldehyde was used in place of bromomethyl derivative. However, the different behaviour of the anil derivative (**3c**) from that of the aminals (**7**, **8**) when undergoing alkaline hydrolysis would also suggest that the latter are not resistant because of the possible iminoether equilibrium that favours the cleavage even under mild conditions (Fig. 4).

A confirmation that this was the case came from the observation that the proton spectrum of compound **8** soon after its recording ^{13}C NMR spectrum, run in $CDCl_3$, presented as a complicated mixture of **8** with the hydrolysis compounds. However, the most important resonances were easily deduced along with the evidence of the aldehyde signals.

The known intermediates necessary for this work are mentioned later in the Section 3. The new intermediates **1b**, **1d**, **5**, **6** were prepared according to Fig. 5. Interestingly bromination of compounds **16**, **17** followed a different course. In the case of **16** we obtained either the monobromo, **1d**, or the dibromo derivative, **18**, whereas in the case of **17** only the monobromo compound (**1b**) was isolated.

3. Experimental

Melting points (m.p.) are uncorrected and were taken in open capillaries on a 510 Buchi apparatus. Infrared spectra, unless otherwise specified, were recorded as Nujol mulls on a Perkin–Elmer 781 spectrometer and are expressed in cm^{-1}. UV spectra are in nm for ethanol solutions and were recorded on a Perkin–Elmer Lambda 5 instrument. Light petroleum refers to the fraction with b.p. 40–60 °C. Elemental analyses (C, H, Cl, N) were performed at the Laboratorio di Microanalisi, Dipartimento di Scienze Farmaceutiche, University of Padova, Italy, and analytical results were within $\pm 0.4\%$ of theoretical values. ^1H and ^{13}C NMR spectra are in ppm (δ) and were recorded at 200 and 50 MHz on a Varian XL-200 instrument. Both reaction progress and product purity were monitored on TLC silica gel plates.

3.1. Intermediates

O-Phenylendiamine was commercially available. 1-Phenyl-2-bromomethylketone and 2-Bromomethyl-3-phenylquinoxaline (**1a**) were obtained as described by Wegman and Dahn [18]. 2-Bromomethyl-3-phenyl-7-trifluoromethylquinoxaline (**1c**) was prepared as described [7]. Diethyl *p*-aminobenzoylglutamate (**2**) was commercially available, while the *N*-methyl derivative (**9**) was prepared by alkylation of **2** with methyl iodide according to the indications of the literature [19].

3.1.1. Preparation of 2-bromomethyl-3-phenyl-6-trifluoromethylquinoxaline (*1b*)

To a solution of 2-methyl-3-phenyl-6-trifluoromethylquinoxaline (**17**) (1 g, 3.46 mmol) [20], and sodium

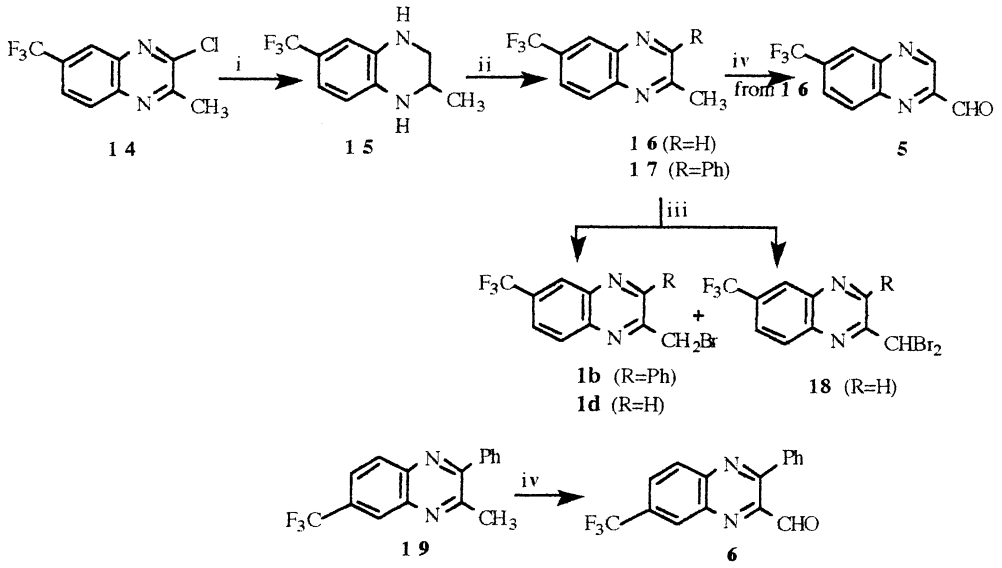

Fig. 5. Preparation of the intermediates **1b**, **d** and **5**, **6**. (i) H_2–Pd/C, TEA at 3 atm; (ii) 30% H_2O_2/H^+; (iii) *N*-bromosuccinimide in CCl_4; (iv) SeO_2 in dioxane.

acetate (0.24 g) in glacial acetic acid (20 ml), bromine (0.55 g, 3.46 mmol) in acetic acid (2 ml) was added within 10 min at room temperature (r.t.) under stirring. Then the mixture was heated at 100 °C for 30 min and during this time the brown solution becomes clear. On cooling, after evaporation under reduced pressure, an orange residue (1.2 g) was obtained and recrystallised from ethanol to give compound **1b** as pale yellow needles (0.6 g, 47%), m.p. 94–96 °C. Analysis for $C_{16}H_{10}BrF_3N_2 \cdot 0.5H_2O$: C, H, N. UV: λ_{max} 329, 241, 206; ^{1}H NMR ($CDCl_3$): δ 8.47 (1H, s, H-5), 8.27 (1H, d, $J = 8.78$ Hz, H-8), 7.97 (1H, dd, $J = 8.78$ and 1.70 Hz, H-7), 7.85–7.75 (2H, m, H-2′,6′), 7.65–7.55 (3H, m, H-3′,4′,5′), 4.77 (2H, s, CH_2).

3.1.2. Preparation of 2-bromomethyl-6-trifluoromethylquinoxaline (*1d*)

*3.1.2.1. 2-Methyl-1,2,3,4-tetrahydro-6-trifluoromethylquinoxaline (**15**).* A solution of **14**, prepared as described [1] (1 g, 4 mmol) in ethanol (40 ml) and in the presence of TEA (0.4 g, 4 mmol) was hydrogenated at 3 atm and 15 °C with 10% palladised charcoal (0.2 g). Within 50 min the take up of hydrogen ceased. The catalyst was filtered off through a filter paper, washed with ethanol and the mother liquors evaporated to dryness. The residue was taken up with water to give **15** as a red orange solid (0.75, 85%), m.p. 85–87 °C from ethanol water. Analysis for $C_9H_{11}F_3N_2$: C, H, N; IR: 3380, 3340; ^{1}H NMR ($CDCl_3$): δ 6.81 (1H, dd, $J = 7.6$ and 1.5 Hz, H-7), 6.68 (1H, d, $J = 1.5$ Hz, H-6), 6.46(1H, d, $J = 7.6$, H-8), 3.80 (2H, br s, NH), 3.54–3.47 (1H, m, H-2), 1.20 (3H, d, $J = 6.34$ Hz, 2-Me).

*3.1.2.2. 2-Methyl-6-trifluoromethylquinoxaline (**16**).* Thirty percent hydrogen peroxide (2 ml) was added to a solution of **15** (0.50 g) in methanol (6 ml) with additional concentrated HCl (3 gtt). The solution darkened with little exothermic reaction while stirred for 30 min. Then, upon dilution with water a solid was filtered off and washed with water to give **16** (0.4 g, 81%) that was recrystallised from ethanol water: m.p. 100–102 °C. Analysis for $C_9H_7F_3N_2$: C, H, N. ^{1}H NMR ($CDCl_3$): δ 8.84 (1H, s, H-3), 8.38 (1H, d, $J_{6,7} = 1.60$ Hz, H-5), 8.14 (1H, d, $J_{7,8} = 8.60$, H-8), 7.92 (1H, dd, $J_{7,8} =$ 8.60 and $J_{6,7} = 1.60$ Hz, H-7), 2.82 (3H, s, Me).

*3.1.2.3. 2-Bromomethyl-6-trifluoromethylquinoxaline (**1d**) and 2-dibromomethyl-6-trifluoromethylquinoxaline (**18**).* A suspension of **16** (0.5 g, 2.36 mmol) and *N*-bromosuccinimide (0.42 g, 2.36 mmol) in the presence of benzoyl peroxide (10 mg) in dry carbontetrachloride (10 ml) was refluxed under irradiation with 300 W lamp for 7 h. Heating was stopped when the mixture became dark brown (after 6 h). On cooling, after filtration of succinimide (0.22 g), the mother liquors were evaporated to dryness to give a semi solid residue (0.65 g) which was purified by flash column chromatography over silica gel eluting with a mixture of light petroleum and ethyl acetate in 98:2 ratio. The fast moving fraction gave on evaporation compound **18** (0.26 g, 15%) as an air sensitive whitish powder: m.p. 130–135 °C. Analysis for $C_{10}H_5Br_2F_3N_2$: C, H, N. ^{1}H NMR ($CDCl_3$): δ 9.46 (1H, s, H-3), 8.46 (1H, s, H-5), 8.19 (d, $J_{7,8} = 8.80$ Hz, H-8), 7.98 (1H, dd, $J_{7,8} = 8.80$ and $J_{5,7} = 2.00$ Hz, H-7), 6.97 (1H, s, $CHBr_2$). Further elution of the column gave a small portion of an inseparable mixture of **1d** and **18** (50 mg) and in the end the most abundant fraction gave

compound **1d** as white powder (0.3 g, 44%), m.p. 90–91 °C (from ethanol as needles). Analysis for $C_{10}H_6BrF_3N_2$: C, H, N. ^{1}H NMR ($CDCl_3$): δ 9.10 (1H, s, H-3), 8.43 (1H, d, $J_{7,5} = 1.40$ Hz, H-5), 8.20 (1H, d, $J_{7,8} = 8.70$ Hz, H-8), 7.97 (1H, dd, $J_{7,8} = 8.70$ and $J_{7,5} = 1.40$ Hz, H-7) 4.75 (2H, s, CH_2).

*3.1.2.4. 6-Trifluoromethylquinoxaline-2-carboxyaldehyde (**5**).* Selenium dioxide (0.38 g, 3.42 mmol) in a mixture of dioxane (1.2 ml) and water (0.25 ml) was added to a solution of **16** (0.5 g, 2.36 mmol) in dioxane (2.5 ml). The resulting suspension when stirring was heated at 75–85 °C for 5.5 h. On cooling, the mixture was filtered and the residue washed with dioxane. The mother liquors were evaporated in vacuo. The brown residue (0.6 g) was purified by silica gel column chromatography eluting with toluene to give **5** as whitish powder (0.2 g, 38% yield), m.p. 137–139 °C. IR: 1720; ^{1}H NMR ($CDCl_3$): δ 10.30 (1H, s, CHO), 9.50 (1H, s, H-3), 8.51 (1H, s, H-5), 8.39 (1H, d, $J_{7,8} = 8.80$ Hz, H-8), 8.06 (1H, dd, $J_{7,8} = 8.80$ and $J_{7,5} = 1.60$ Hz, H-7).

*3.1.2.5. 3-Phenyl-7-trifluoromethylquinoxaline-2-carboxyaldehyde (**6**).* A suspension of **19** (2 g, 6.94 mmol) and an excess of selenium dioxide (2 g, 18 mmol) in ethyl acetate (20 ml) was stirred under reflux for 18 h. On cooling, the mixture was filtered through paper and the mother liquors were evaporated under reduced pressure. A brown red residue (1.9 g) was obtained and purified from eptane and further flash chromatography eluting with a mixture of light petroleum and ethyl acetate (95:5) to give compound **6** (1.5 g, 71%) m.p. 126–127 °C from eptane. Analysis for $C_{16}H_9F_3N_2O$: C, H, N. IR: 1710; UV: λ_{max} 322, 237, 206; ^{1}H NMR ($CDCl_3$): δ 10.34 (1H, s, CHO), 8.64 (1H, s, H-5), 8.35 (1H, d, $J = 8.80$ Hz, H-8), 8.10 (1H, dd, $J = 8.80$ and 1.98 Hz, H-7), 7.69–7.65 (2H, m, H-2′,6′), 7.64–7.56 (3H, m, H-3′,4′,5′).

*3.1.3. General procedure for preparation of Schiff bases (**3a–d**) and related compounds (**7**, **8**)*

A solution of 2-bromomethylquinoxaline (**1a–d**) (2.72 mmol) and diethyl *p*-aminobenzoylglutamate (**2**) (5.44 mmol) in dry DMA (20 ml) was stirred at r.t. for 72 h. The dark brown solution was evaporated in vacuo with gentle heating. An oily residue, impure at TLC, was taken up with methanol to give a solid that was recrystallised or submitted to further purification by flash chromatography as indicated below.

Compound **3a** (62% yield) m.p. 200–202 °C from ethanol. Analysis for $C_{31}H_{30}N_4O_5$: C, H, N. IR: 3330, 1750, 1730, 1640; UV: λ_{max} 380, 309, 284, 250, 204; ^{1}H NMR ($CDCl_3$): δ 9.72 (1H, s, CH=N), 8.50–7.58 (13H, m, arom), 7.41 (1H, d, $J = 7.32$ Hz, N*H*–CH), 5.05–4.90 (1H, m, NH–C*H*–CH_2–), 4.31 (2H, q, CH_2–Me), 4.18 (2H, q, CH_2–Me), 2.70–2.20 (4H, m, CH_2–CH_2), 1.37 and 1.25 (3H, t, Me).

Compound **3b** (45% yield), m.p. 247–250 °C from methanol. Analysis for $C_{32}H_{29}F_3N_4O_5$: C, H, N. IR: 3330, 1750, 1730, 1640; UV: λ_{max} 384, 305, 287, 252, 204; ^{1}H NMR ($CDCl_3$): δ 9.66 (1H, s, CH=N), 8.76 (1H, s, H-5), 8.48 (1H, d, $J = 8.98$ Hz, H-8), 8.44–8.27 (5H, m, arom), 8.05 (1H, dd, $J = 8.90$ and 1.80 Hz, H-7), 7.68–7.59 (4H, m, arom), 7.52 (1H, d, N*H*–CH), 4.94 (1H, st, NH–C*H*), 4.32 and 4.20 (2H, q, CH_2–Me), 2.74–2.17 (4H, m, CH_2–CH_2), 1.37 and 1.27 (3H, t, Me).

*3.1.3.1. Diethyl N-[4-(6-trifluoromethylquinoxalin-2-yl)(methoxymethyl)]amino-benzoyl-L-glutamate (**7**).* The aldehyde (**5**) (0.1 g, 0.44 mmol) was closely mixed in a mortar with an excess of **2** (0.32 g, 0.99 mmol) and then the mixture heated in vacuo at 130 °C for 40 min. On cooling, The melted mass was taken up with methanol and left on standing. Compound **7** was obtained as a brown solid (0.15 g, 60%), m.p. 124–126 °C. Analysis for $C_{27}H_{29}F_3N_4O_6$: C, H, N. IR: 3280, 3180, 1720, 1670, 1630; UV: λ_{max} 330, 273, 203; ^{1}H NMR ($CDCl_3$): δ 9.24 (1H, s, H-3H), 8.50 (1H, s, H-5), 8.30 (1H, d, $J_{7,8} = 8.40$ Hz, H-8), 8.02 (1H, dd, $J_{7,8} =$ 8.40 and $J_{5,1} = 1.60$ Hz, H-7), 7.78 (2H, d, $J_{2',3'} = 8.40$ Hz, H-3′,5′), 7.04 (2H, d, $J_{2',3'} = 8.40$ Hz, H-2′,6′), 6.88 (1H, d, $J = 7.40$ NH–CO, collapses with D_2O), 6.36 (1H, d, $J = 7.00$, N*H*–CH), 6.22 (1H, d, $J = 7.00$, H-9), 4.80 (1H, m, CH_2–C*H*–NH), 4.24 and 4.11 (2H, q, *O*-CH_2–Me), 3.15 (3H, s, OMe), 2.60–2.20 (4H, m, CH_2CH_2), 1.31 and 1.23 (3H, t, Me).

*3.1.3.2. Diethyl N-[4-(3-phenyl-7-trifluoromethylquinoxalin-2-yl)methoxymethyl] aminobenzoyl-L-glutamate (**8**).*

i) A mixture of **6** (0.3 g, 0.99 mmol) and **2** (0.64 g, 1.98 mmol) was melted at 125–130 °C in vacuo (0.5–0.7 mmHg) On cooling, the glassy residue was dissolved in methanol and after a few hours crystallised as orange–yellow leaflets (0.26 g, 42% yield): 132–134 °C. Analysis for $C_{33}H_{33}F_3N_4O_6$: C, H, N. IR: 3350, 3330, 1750, 1720, 1630, 1610; UV: λ_{max} 320, 289, 280, 237, 204; ^{1}H NMR ($CDCl_3$): δ 8.56 (1H, s, H-8), 8.31 (1H, d, $J_{5,6} = 8.92$ Hz, H-5), 8.01 (1H, dd, $J_{8,6} = 1.90$ Hz and $J_{5,6} = 8.92$ Hz, H-7), 7.83 (2H, m, H-2′,6′), 7.70 (2H, d, $J_{2'',3''} = 8.54$ Hz, H-3″,5″), 7.66–7.58 (3H, m, 3′,4′,5′), 6.83 (2H, d, $J = 8.54$ Hz, H-2″,6″), 6.25 (1H, s, C*H*–OMe), 4.76 (1H, m, NH–C*H*–CH_2), 4.25 and 4.10 (2H, q, *O*-CH_2–OMe), 3.30 (3H, s, OMe), 2.55–2.04 (4H, m, CH_2 CH_2), 1.30 and 1.22 (3H, t, Me).

^{13}C NMR ($CDCl_3$): δ 173.26 (s, C=O), 172.22 (s, C=O), 166.70 (s, C=O), 158.31 (d), 148.02(d), 80.4 (d, C-9).

ii) In a flask equipped with Dean Stark apparatus a solution of equimolar amounts (2.65 mmol) of **6** (0.8 g) and **2** (0.85 g) in benzene (15 ml) was refluxed for 20 h. On cooling, the solvent was removed under vacuum to give a glassy solid that once taken up with methanol on standing yielded **8** (0.55 g, 34%) identical to the above specimen.

iii) In an identical run as above but heating the reactants in refluxing dry benzene over activated (5 Å) molecular sieves for 6 h, the work up of the reaction gave a similar result. However, the attempts of isolation of the Schiff base (**3c**) either using different solvents or flash chromatography, were unsuccessful. In the last case the starting reactants were recovered after elution.

*3.1.3.3. N-[1-(3-Phenyl-7-trifluoromethylquinoxalin-2-yl)methyl] methylaniline (**10**).* A solution of **1c** (0.2 g, 0.55 mmol) and an excess of *N*-methylaniline (0.12g, 1.1 mmol) in dry DMA (4 ml) was stirred at r.t. for 72 h. After removal of the solvent in vacuo with gentle heating a crude oily residue was taken up with methanol and induced to crystallise. A solid was then collected (0.1 g), washed with hot methanol and eventually dried, to give **10**, m.p. 105–107 °C. Analysis for $C_{23}H_{16}F_3N_3$: C, H, N. ^{1}H NMR ($CDCl_3$): δ 8.39 (1H, s, H-8), 8.24 (1H, d, $J_{5,6} = 8.88$ Hz, H-5), 7.92 (1H, dd, $J_{5,6} = 8.88$ and $J_{6,8} = 1.76$ Hz, H-6), 7.60–7.48 (5H, m, arom), 7.20–7.00 (2H, m, arom), 6.70–6.55 (3H, m, arom), 4.85 (2H, s, CH_2), 3.00 (3H, s, Me).

*3.1.3.4. N-[4-(3-Phenyl-7-trifluoromethylquinoxalin-2-yl)methyl]-imino–p-toluidine (**11**).*

i) Equimolar amounts (1.36 mmol) of **1c** (0.5 g) and *p*-toluidine (0.14 g) in dry DMA (10 ml) were heated at 100 °C under stirring. On evaporation of the solvent in vacuo a residue was taken up with methanol to give **11**, as a yellow solid, that were collected (0.30 g, 57%). An analytical sample was recrystallised from ethanol: yellow leaflets m.p. 162–163 °C. Analysis for $C_{23}H_{16}F_3N_3$: C, H, N, UV: λ_{max} 291, 237, 202; ^{1}H NMR ($CDCl_3$): δ 8.80 (1H, s, CH=N), 8.72 (1H, s, H-8), 8.31 (1H, d, $J_{5,6} = 8.60$ Hz, H-5), 8.01 (1H, dd, $J_{5,6} = 8.60$ and $J_{6,8} = 1.76$ Hz, H-6), 7.83–7.73 (2H, m, arom), 7.65–7.56 (3H, m, arom), 7.22 (4H, m, arom), 2.34 (3H, s, Me); ^{13}CNMR ($CDCl_3$): δ 121.52 (d, C-9).

ii) A mixture of aldehyde (**6**) (0.1 g, 0.33 mmol) and an excess of *p*-toluidine (0.66 mmol) was melted at 110 °C in vacuo for 7 min. Formation of water was observed. On cooling, the solid mass was powdered in a mortar and gave **11** (0.11 g, 77%) identical (m.p.; UV; ^{1}H NMR) to the above authentic specimen.

*3.1.3.5. N-[4-(3-Phenyl-7-trifluoromethylquinoxalin-2-yl)methylimino]benzoate (**12**).* A solution of equimolar amounts of **1c** (0.5 g, 1.36 mmol) and ethyl *p*-aminobenzoate (0.125 g, 1.36 mmol) in dry DMA (10 ml) was heated at 100 °C under stirring. The solution soon becomes blue–green. On cooling, a little amount of an unidentified solid was filtered off and the mother liquors evaporated in vacuo. A yellow orange semisolid residue was taken up with methanol to give yellow crystals of **12** (0.2 g, 33%). An analytical sample was recrystallised from ethanol: m.p. 198–200 °C. Analysis for $C_{25}H_{18}F_3N_3O_2$: C, H, N. IR: 1720; UV: λ_{max} 381, 288, 251, 204. ^{1}H NMR ($CDCl_3$): δ 9.86 (1H, s, CH=N), 8.64 (1H, s, H-8), 8.52 (3H, a d, $J_{5,6} = 9.08$ Hz, H-2″,6″+H-5), 8.37–8.25 (4H, m, H-7+3″,5″), 8.15 (1H, dd, $J_{5,6} =$ 9.08 and $J_{6,8} = 1.76$ Hz, H-6), 7.70–7.60 (3H, m, 3′,4′,5′), 4.54 (2H, q, CH_2–Me), 1.54 (3H, t, CH_2–*Me*). The methanolic mother liquors on evaporation gave an oily residue (0.4 g) that was constituted of starting materials.

*3.1.3.6. N-[4-(3-Phenyl-6-trifluoromethylquinoxalin-2-yl)methylimino]benzoate (**13**).* In a similar fashion as above compound (**13**) was obtained in 41% yield. M.p. 180–182 °C from methanol. Analysis for $C_{25}H_{18}F_3N_3O_2$: C, H, N. IR: 1720; UV: λ_{max} 384, 286, 253, 230, 204. ^{1}H NMR ($CDCl_3$): δ 9.92 (1H, s, CH=N), 8.79 (1H, d, $J_{5,7} = 1.90$, H-5), 8.57 (2H, dd, $J_{2'',3''} = 8.50$ and $J_{2'',6''} = 1.98$ Hz, H-2″,6″), 8.49 (2H, dd, $J_{7,8} = 8.92$ and $J_{5,7} = 1.98$ Hz, H-7), 8.37–8.30 (3H, m, H-2′,6′+H-8), 8.07 (2H, dd, $J_{5'',6'} = 8.50$ and $J_{2'',6''} = 1.98$ Hz, H-3″,5″), 7.70–7.60 (3H, m, H-3′,4′,5′), 4.54 (2H, q, CH_2–Me), 1.54 (3H, t, CH_2–*Me*).

4. Enzymology

4.1. Materials and methods

Thymidylate synthase from *L. casei* and *L. major*, human dihydrofolate reductase. The enzymatic assays have been run on spectrophotometer UV–Vis, Beckman DU 640, equipped with a thermostated circulating bath HAAKE F3C.

N^5,N^{10}-methylene tetrahydrofolate is a generous gift of Eprova (Schaffhausen, Switzerland). The plasmids were supplied by Dr. Santi (University of California, San Francisco, USA) and Dr. Maley (New York State Department of Health, New York).

4.2. Experimental

Plasmids that express *L. casei* TS (TS, EC 2.1.1.45) in the Thy—*Escherichia coli* strain X2913 have been described [22]. The enzyme has been purified by column chromatography method using phosphocellulose (P11,

Biorad) and hydroxylapatite (HAP, Biorad) resin, with phosphate buffer as eluent [23]. Human thymidylate synthase (hTS) and *L. major* thymidylate synthase (LmTS) has been purified as reported using affinity column chromatography [24–26]. The enzyme preparations were >95% homogeneous as shown by SDS-polyacrylammide gel electrophoresis. The purified enzymes have been stored at −80 °C in 10 mM phosphate buffer, pH 7.0, 0.1 mM EDTA until use.

The activity of TS has been determined by steady state kinetic analysis, spectrophotometrically, by following the increasing absorbance at 340 nm due to the oxidation reaction of N^5,N^{10}-methylene tetrahydrofolate to dihydrofolate at 5,6 bond. 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 formaldheyde, 1 mM EDTA and 75 mM 2-mercaptoethanol. One ml of reaction mixture is formed by standard TES buffer pH 7.4, dUMP 120 μM, 6-(*R*,*S*)-1-CH_2CH_4-folate 180 μM, enzyme 0.07 μM [27].

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.

TS inhibition studies were run in the general condition of standard TS assays with the exception of folate concentration kept at concentration of times the Km value.

The assay conditions are those of the standard TS assay.

Human dihydrofolate reductase, hDHFR (DHFR; EC1.5.1.3) has been purified through alternate steps of column chromatography and ammonium sulfate precipitations as reported [28].

DHFR (EC 1.5.1.3) catalyses the NADPH dependent reduction of dihydrofolate (H_2folate) to tetrahydrofolate (H_4folate). 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 1 nmol of H_2folate reduced per minute.

The reaction mixture was formed by: 50 μM TES, pH 7.0, EDTA 1 μM, 1-mercaptoethanol 75 μM, NADPH 100 μM, H_2folate 58.03 μM [29].

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

Assuming a competitive inhibition pattern Ki was calculated based on the experimental percentage of inhibition of the reported concentration due to the limited solubility.

4.3. Results of enzymatic assays and discussion

From the data of Table 1 only compound **Ie** showed a significant inhibition activity (74%) against hDHFR at 10 μM with a Ki of 200 nM. In this test also compound **3c** exhibits 20% inhibition value at the same concentration, the other compounds being inactive at 10 μM. A striking difference of both activity and selectivity was observed in TS test of different origin. All compounds seem to bind rather to LcTS than LmTS or hTS in the range 3–10 μM. Among these compound **3c** showed comparable inhibition constant of 2.2–4 μM Ki between LcTs and hTS and hDHFR.

Table 2
−log GI_{50}, −log TGI, −log LC_{50} mean graph midpoints (MG-MID) [a] of in vitro inhibitory activity test for compounds **3a**–**c**, **8** against human tumour cell lines [b]

Compound	−log GI_{50} = μM	−log TGI	−log LC_{50}
3a	4.04 = 91.26	4.00	4.00
3b	4.28 = 52.48	4.04	4.00
3c	4.28 = 52.48	4.09	4.01
8	4.93 = 11.74	4.56	4.22

[a] (MG-MID) mean graph midpoints, the average sensitivity of all cell lines towards the test agent.
[b] From NCI.

Table 1
Enzymatic inhibition shown by compounds **3b**, **3c**, **8**, **11**, **12**, **13**, **Ie** against LcTS, LmTS, hTS and DHFR

Compound	LcTS		LmTS		hTS		human DHFR	
	I%	Ki	I%	Ki	I%	Ki	I%	Ki
3b	20 (3)	9	6 (10)	68	NI (10)		NI (10)	
3c	38 (10)	4	3 (10)	140	24 (10)	3	20 (10)	2.2
8	NI.(10)		3 (10)	140	NI (10)		NI (10)	
11	26 (5)	4	9 (10)	44	5 (10)		NI (10)	
12	NI.(10)		5 (10)	83	NI (10)		NI (10)	
13	38 (3)	4	12 (10)	35	3 (10)	34	NI (10)	
Ie			NI (50)		2 (50)	258	74 (10)	0.2

The percentage of inhibition at the μM concentration indicated in parenthesis and the calculated Ki values are shown.

Table 3
Percent tumour growth inhibition recorded on subpanel cell lines at 10^{-5} and 10^{-4} M concentration of compounds **3a**–**c**, **8**

Subpanel cell lines	**3a**		**3b**		**3c**		**8**	
	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}
Leukaemia								
CCRF-CEM	*	*	*	*	*	*	137	151
HL-60(TB)	*	*	*	*	*	59	163	180
K-562	*	*	46	*	*	*	115	182
MOLT-4	*	*	*	*	*	*	127	152
RPMI-8226	*	*	*	*	*	*	71	110
SR	*	*	*	*	28	*	99	98
Non small cell lung cancer								
A549/ATCC	*	*	*	*	*	49	*	162
EKVX	*	*	*	*	*	*	*	195
HOP18	*	78	37	67	*	*	*	129
HOP-62	*	*	30	115	*	149	*	198
HOP-92	*	*	*	*	41	130	*	191
NCI-H226	*	*	*	*	*	*	*	183
NCI-H23	*	*	*	*	*	78	*	177
NCI-H322M	*	*	*	*	28	*	*	200
NCI-H460	*	*	*	*	*	*	44	194
NCI-H522	*	*	*	43	*	96	38	199
LXFL 529	*	*	*	45	*	*	51	194
Small cell lung cancer								
DMS 114	*	*	*	75	*	*	41	153
DMS 273	*	*	*	77	*	*	*	175
Colon cancer								
COLO 205	*	*	*	*	*	*	*	145
DLD-1		*	*	*	*	*	58	195
HCC-2998	*	*	26	81	*	43	*	199
HCT-116	*	*	25	76	*	68	91	200
HCT-15	*	*	*	*	*	*	36	144
HT29	*	*	*	*	*	*	55	182
KM12	*	*	*	*	*	*	61	191
KM20L2		*	30	72	*	*	*	198
SW-620	*	*	*	*	*	*	67	184
SNC cancer								
SF-268	*	*	29	*	31	83	*	197
SF-295	*	*	*	99	21	55	*	181
SF-539	*	*	47	147	59	149	*	200
SNB-19	*	43	*	87	53	146	37	200
SNB-75	35	84	82	105	90	157	*	186
SNB-78	*	40	*	66	33	100	*	174
U251	*	45	*	98	50	116	*	87
XF 498	*		*	*	95	169	*	194
Melanoma								
LOX IMVI	*	*	*	*	*	*	*	197
MALME-3M	*	*	*	*	*	*	*	197
M14	*	53	*	60	*	48	*	194
M19-MEL	*	*	*	*	*	*	*	184
SK-MEL-2	*	*	*	136	*	71	*	183
SK-MEL-28	*	*	*	*	*	*	*	197
SK-MEL-5	*	*	*	65	*	48	*	200
UACC-257	*	*	*	*	*	*	*	180
UACC-62	*	*	43	113	*	59	*	161
Ovarian cancer								
IGROV1	*	*	*	73	*	*	*	162
OVCAR-3	*	*	*	47	*	*	*	182
OVCAR-4	*	*	*	42	*	*	*	108
OVCAR-5	*	*		*	*	*	*	199
OVCAR-8	47	66	*	*	*	*	38	139
SK-OV-3	*	*	*	90	*	148	*	186

Table 3 (*Continued*)

Subpanel cell lines	**3a**		**3b**		**3c**		**8**	
	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}
Renal cancer								
786-0	*	49	46	97	*	75	*	179
A498	*	*	*	79	*	112	*	172
ACHN	*	*	*	76	*	65	*	193
CAKI-1	*	*	*	*	*	*	*	197
RXF 393	*	56	131	*	79	147	*	197
RXF 631	*	*	*	*	*	*	*	194
SN12C	*	55	*	*	*	*		200
TK-10	*	*	*	120	*	127	*	198
UO-31	*	*	*	*	*	*	*	200

*, Below 20 and 40% growth inhibition at 10^{-5} and 10^{-4} M concentration, respectively.

In conclusion we can say that in anticancer screening the similarity of **8** and **Ie** with methotrexate is apparently a good point to explain the best activity recorded in these tests. Unfortunately compound **Ie** was not selected for anticancer screening by NCI.

5. Pharmacology

Evaluation of anticancer activity was performed on four compounds inconvertibly selected by National Cancer Institute of Bethesda referring to structures **3a**–**c**, **8** of Fig. 2, following the known [21] in vitro disease-oriented antitumour screening program against a panel of 60 human tumour cell lines. 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 2 the response parameters GI_{50}, TGI and LC_{50} refer to the concentration of the agent in the assay that produced 50% growth inhibition, total growth inhibition, 50% cytotoxicity, respectively and are expressed as mean graph midpoints. In Table 3 we reported the activities of those compounds which showed percent growth inhibition greater than 40% on subpanel cell lines at 10^{-5} and 10^{-4} M concentration.

5.1. Results of the in vitro pharmacological anticancer assays

The data of in vitro anticancer activity reported in Table 2 established that the average sensitivity of all cell lines towards the tested agents, represented as mean graph midpoints, falls in the range 11.74–91.20 μM concentrations, where the TGI is very close to LC_{50} for **3a** and **3b**. Compounds **3b** and **3c** exhibited identical GI_{50} and a slight difference between TGI and LC_{50} values, while compound **3a** appears almost completely inactive at the lowest concentrations. From the data of Table 3 it is evident that the most active compound was **8**, which at 10^{-4} M exhibited the largest sensitivity upon all subpanel cell line (60 over 60) and at a lower concentration the activity was maintained over 18 cell lines at a relevant degree of percent growth inhibition mainly against leukaemia and colon subpanel cell lines. Compound **3c** at 10^{-4} M was active over 26 cell lines while at 10^{-5} M the sensitivity was maintained for all subpanel SNC cancer cell lines.

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References

[1] M. Loriga, M. Fiore, P. Sanna, G. Paglietti, Quinoxaline chemistry: Part 4. 2-(*R*)-Anilinoquinoxalines as non classic antifolate agents. Synthesis, structure elucidation and evaluation of in vitro anticancer activity, Farmaco 50 (1997) 289–301.
[2] M. Loriga, M. Fiore, P. Sanna, G. Paglietti, Quinoxaline chemistry. Part 5. 2-(*R*)-Benzymamminoquinoxalines as non classical antifolate agents. Synthesis and evaluation of in vitro anticancer activity, Farmaco 51 (1996) 559–568.
[3] M. Loriga, S. Piras, P. Sanna, G. Paglietti, Quinoxaline chemistry. Part 7. 2-[Aminobenzoates]- and 2[Aminobenzoylglutamate]-quinoxalines as classical antifolate agents. Synthesis and evaluation of in vitro anticancer, anti-HIV and antifungal activity, Farmaco 52 (1997) 157–166.
[4] M. Loriga, P. Moro, P. Sanna, G. Paglietti, Quinoxaline chemistry. Part 8. 2-[Anilino]-3-[carboxy]-6(7)-substituted quinoxalines as non classical antifolate agents. Synthesis and evaluation of in vitro anticancer, anti-HIV and antifungal activity, Farmaco 52 (1997) 531–537.
[5] G. Vitale, P. Corona, M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 9. Quinoxaline analogues of TMQ and 10-propargyl-5,8-dideaza folic acid and its precursors. Synthesis

and evaluation of in vitro anticancer activity, Farmaco 53 (1998) 139–149.
[6] G. Vitale, P. Corona, M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 10. Quinoxaline 10-oxa analogues of TMQ and 10-propargyl-5,8-dideaza folic acid and its precursors. Synthesis and evaluation of in vitro anticancer activity, Farmaco 53 (1998) 150–159.
[7] P. Corona, G. Vitale, M. Loriga, G. Paglietti, M.P. Costi, 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, Farmaco 53 (1998) 480–493.
[8] G. Vitale, P. Corona, M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 12. 3-Carboxy-2[phenoxy]-6(7)substituted quinoxalines and *N*-[4-(6(7) substituted-3-carboxyquinoxaline-2-yl)hydroxy]benzoylglutamates. Synthesis and evaluation of in vitro anticancer activity, Farmaco 53 (1998) 594–601.
[9] P. Corona, G. Vitale, M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 13. 3-Carboxy-benzylamino-substituted quinoxalines and *N*-[4-[3-carboxyquinoxaline-2-yl)aminomethyl]benzoyl-L-glutamate: synthesis and evaluation of in vitro anticancer activity, Farmaco 55 (2000) 77–86.
[10] P. Corona, G. Vitale, M. Loriga, S. Alleca, G. Paglietti, Pirrolo[1,2-*a*]quinoxalines analogues of antifolic trimetrexate and methotrexate. Abstracts of XVIth International Symposium on Medicinal Chemistry, Bologna, 18–22 September 2000, p. 526.
[11] S. Piras, M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 14. 4-(2-Quinoxalylamino)-phenylacetates and 4-(2-quinoxalylamino)-phenylacetyl-L-glutamates as analogues–homologues of classical antifolate agents. Synthesis and evaluation of in vitro anticancer activity, Farmaco 57 (2002) 1–8.
[12] S. Piras, M. Loriga, G. Paglietti, Quinoxalines analogues–homologues of methotrexate, Abstracts of Hungarian–German–Italian–Polish Joint Meeting on Medicinal Chemistry (HGIP JMMC), Budapest, 2–6 September 2001, p. 141.
[13] P. Corona, M. Loriga, G. Paglietti, P. La Colla, M.G. Setzu, R. Loddo, Imidazo[1,2-*a*]- and 1,2,4-triazolo[4,3-*a*]quinoxalines analogues of antifolic trimetrexate and methotrexate. Abstracts of Hungarian–German–Italian–Polish Joint Meeting on Medicinal Chemistry (HGIP JMMC), Budapest, 2–6 September 2001, p. 64.
[14] G. Vitale, M. Loriga, G. Paglietti, M.P. Costi, A. Venturelli, F. Marturana, L. Marongiu, P. La Colla, Quinoxalines analogues of thymitaq and 2-(arylthio)quinoxalines analogues of trimetrexate and methotrexate. Abstracts of Hungarian–German–Italian–Polish Joint Meeting on Medicinal Chemistry (HGIP JMMC), Budapest, 2–6 September 2001, p. 184.
[15] H.O. Kalinoski, S. Berger, S. Braun, Carbon-13 NMR Spectroscopy (and references cited therein), Wiley, 1991, p. 241.
[16] G.B. Barlin, The pyrazines, in: A. Weissberger, E.C. Taylor (Eds.), The Chemistry of Heterocyclic Compounds (and references cited therein), Wiley, New York, 1982, p. 147.
[17] G.W.H. Cheeseman, R.F. Cookson, Condensed pyrazines, in: A. Weissberger, E.C. Taylor (Eds.), The Chemistry of Heterocyclic Compounds (And references cited therein), Wiley, New York, 1979, p. 210.
[18] J. Wegman, H. Dahn, Uber den Einfluss von Nachbarsubstituenten auf di Additionsfahigkeit von Carbonylgruppen, Helv. Chim. Acta XXIX (1946) 1247.
[19] T.R. Jones, T.J. Thornton, A. Flinn, A.L. Jackman, D.R. Newell, A.H. Calvert, Quinazoline antifolate inhibiting TS: 2-desamino derivatives with enhanced solubility and potency, J. Med. Chem. 32 (1989) 847–852.
[20] (a) M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 1. 6-Trifluoromethylquinoxalines and their N-oxides. Synthesis and structure elucidation, J. Chem. Res. (S) (1986) 16–17.(b) M. Loriga, G. Paglietti, Quinoxaline chemistry. Part 1. 6-Trifluoromethylquinoxalines and their N-oxides. Synthesis and structure elucidation, J. Chem. Res. (M) (1986) 277–296.
[21] M.R. Boyd, Status of the NCI Preclinical Antitumor Drug Discovery Screen in Principles and Practice of Oncology, vol. 3, (10) 1989, pp. 1–12.
[22] S. Climie, D.V. Santi, Chemical synthesis of the thymidylate synthase genes, Proc. Natl. Acad. USA 87 (1990) 633–637.
[23] J.T. Kealy, D.V. Santi, Purification methods for recombinant *L. casei* thymidylate synthase and mutants. A general automated procedures. Protein expression purification 4 (1992) 380–385.
[24] J. Ju, J. Pedersen-Lane, F. Maley, E. Chu, PNAS 96 (1999) 3769–3774.
[25] K. Banerjee, L. Lee, Jr., C. Bennet, R.W. Brockman, B.P. Sani, C. Temple, Jr., A convenient procedure for purification of thymidylate synthase from L1210 cells, Anal. Biochem. 121 (1982) 275–280.
[26] R. Grumont, W. Sirawaraporn, D.V. Santi, Heterologous expression of the bifunctional thymidylate synthase–dihydrofolate reductase from *Leishmania major*, Biochemistry 27 (1988) 3776–3784.
[27] D.V. Santi, P.V. Danenberg, in: R.L. Blacket, S.J. Benkovic (Eds.), Folate and Pteridines. Chemistry and Biochemistry of Folates, vol. 11, Wiley, New York, 1984, pp. 345–399.
[28] J.G. Dann, G. Ostler, R.A. Bjur, W.R. King, P. Scudder, G.C.K. Roberts, S.V. Burgen, Large-scale purification and characterization of dihydrofolate reductase from a methotrexate resistant strain of *Lactobacillus casei*, Biochem. J. 157 (1976) 559–571.
[29] T.D. Meek, E.P. Garvey, D.V. Santi, Purifcation and characterisation of bifunctional T. Thymidylate synthetase–dihydrofolate reductase from methotrexate-resistant *Leismania tropicae*, Biochemistry 24 (1985) 678–686.