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Bioorganic & Medicinal Chemistry 14 (2006) 7846–7853
Bioorganic & Medicinal Chemistry

Design, synthesis, and biological evaluation of substituted 2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-ylamine related compounds as fructose-1,6-bisphosphatase inhibitors

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Received 24 March 2006; revised 26 July 2006; accepted 28 July 2006

Abstract—In a search for structurally new inhibitors of fructose-1,6-bisphosphatase (F16BPase), substituted 2,3-dihydro-1*H*-cyclopenta[*b*]quinoline derivatives were synthesized. It has been shown that the 2,3-dihydro-1*H*-cyclopenta[*b*]quinoline moiety may represent a suitable scaffold for the synthesis of potent F16BPase inhibitors endowed with significantly lower EGFR tyrosine kinase inhibitory activity.

1. Introduction

Type 2 diabetes is a heterogeneous disorder characterized by hyperglycemia. The liver produces excessive amounts of glucose through the gluconeogenesis and glycogenolysis pathways that, as a consequence, lead to elevated glucose levels characteristic of the disease.[1,2] Although it is not clear yet which of the two routes is the most important,[2] it has been demonstrated that fructose-1,6-bisphosphatase (F16BPase), an enzyme that is predominantly expressed in the liver and kidney, is one of the rate-limiting enzymes of hepatic gluconeogenesis.[3–5] Furthermore, liver F16BPase is elevated in insulin-deficient[6] and insulin-resistant[7] animal models of diabetes, outlining the importance of this enzyme in the control of blood glucose.

Clearly, inhibitors of F16BPase would represent a useful therapy for the treatment of type 2 diabetes because they would be expected to reduce hepatic glucose output and lower blood glucose by inhibiting the elevated rate of gluconeogenesis that is present in diabetic patients.[3,8–10] In 2001, Wright et al.[11] reported on a series of anilinoquinazolines as allosteric inhibitors of F16BPase, previously described as inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase. Anilinoquinazolines represent the first low molecular weight inhibitors of F16BPase that are not fructose or purine phosphates or phosphonates.[11,12]

One year later, a related and detailed structure–activity relationship study by the same research group[13] led to compounds, like the anilinoquinazoline **1** (Table 1), with preferential F16BPase inhibitory activity relative to EGFR tyrosine kinase inhibitory activity. Since the introduction of substituents at the 2-position of the quinazoline moiety of **1** resulted in inhibitors with increased selectivity for F16BPase, we decided to replace the bicyclic quinazoline system of these inhibitors with a tricyclic moiety to verify whether the increased steric hindrance of the resulting inhibitors would positively affect not only the affinity but also the selectivity for F16BPase over EGFR tyrosine kinase.

Thus, the pyrimidine ring of **1** was replaced by a tetrahydroquinoline moiety affording **3**, which bears in its structure the functionalities of **1** relevant for the interaction with the enzyme; that is, the two ethoxy groups, the substituted aniline moiety, and the pyridine basic nitrogen atom. To achieve a better insight into the effect produced by the size of the additional aliphatic ring of **3** on the

Keywords: Fructose-1,6-bisphosphatase inhibitors; Type 2 diabetes; Cyclopenta[*b*]quinoline derivatives; Allosteric inhibitors; Anilinoquinazolines.
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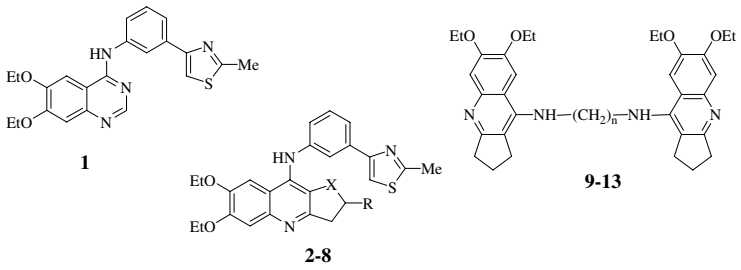
doi:10.1016/j.bmc.2006.07.059

Table 1. Inhibition, expressed as IC_{50} value, of F16BPase, EGFR tyrosine kinase, and AChE by 2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-ylamine related compounds **2**–**13** in comparison with the quinazoline derivative **1**



Compound	X	R	*n*	F16BPase[a] (IC_{50}, μM)	EGFR[b] (IC_{50}, μM)	AChE[c] (IC_{50}, μM)
1				4.41 ± 0.11	11.92 ± 0.95	>10[g]
2	CH_2	H		2.60 ± 0.51	19.74 ± 1.77	>10
3	$(CH_2)_2$	H		10.91 ± 0.30	n.d.[f]	n.d.
4	$(CH_2)_3$	H		8.51 ± 0.41	n.d.	n.d.
5	$CHCH_2NHAc$	H		>100[d]	>100	n.d.
6	$CHCH_2NH_2$	H		>100	n.d.	n.d.
7	CH_2	CH_2NHAc		21.71 ± 2.21	n.d.	n.d.
8	CH_2	CH_2NH_2		29.50 ± 0.10	>100	n.d.
9			3	>100	n.d.	>10
10			4	20%[e]	n.d.	35%[h]
11			5	3.01 ± 0.40	101.62 ± 8.13	13.01 ± 0.34
12			6	>100	n.d.	2.21 ± 0.12
13			7	>100	n.d.	0.177 ± 0.011
Tacrine						0.431 ± 0.012

[a] Rabbit enzyme. Values are means ± SD of at least three experiments.
[b] EGFR tyrosine kinase from A431 cells. Values are means ± SD of at least two independent experiments.
[c] Human recombinant AChE from human serum. Values are means ± SD of at least two independent experiments.
[d] An IC_{50} >100 indicates that no inhibition was noted in the dose response curve up to 100 μM.
[e] Percent inhibition observed at a screening concentration of 30 μM.
[f] Not determined.
[g] An IC_{50} > 10 indicates that no inhibition was noted up to 10 μM.
[h] Percent inhibition observed at a screening concentration of 10 μM.

inhibitory activity of F16BPase, lower (**2**) and higher (**4**) homologs were investigated as well. Since polar substituents at position 2 of the quinazoline moiety of **1** improved the biological profile towards F16BPase, a methylamine and the corresponding acetylated moiety were inserted into the cyclopentene ring of **2**, leading to derivatives **5**–**8**.

It was reported that anilinoquinazolines related to **1** bind as head-to-tail π-stacked dimers at a symmetric allosteric binding site of F16BPase.[13] On this basis, it was argued that the close proximity of the two inhibitor molecules at the binding site may serve as a point of departure to design more potent and selective F16BPase inhibitors. To further validate the hypothesis of Wright et al.,[13] we included in this study bis-dihydrocyclopenta[*b*]quinolines **9**–**13** in which the length of the spacer was varied from three to seven methylene units.

All the reported compounds were assayed for their inhibitory activity against F16BPase and EGFR tyrosine kinase using quinazoline **1** as reference compound.

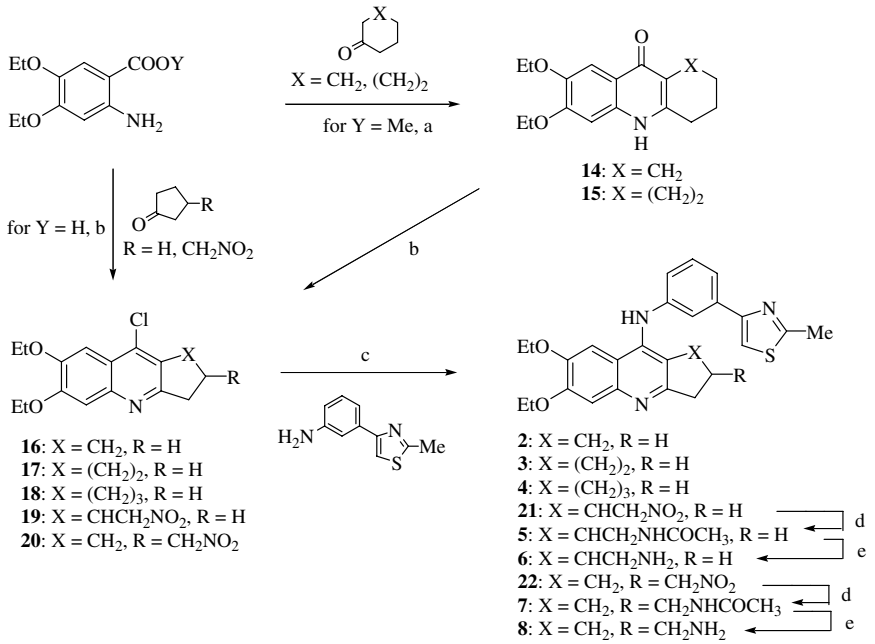
1.1. Chemistry

All the compounds were synthesized by standard procedures (Schemes 1 and 2) and were characterized by IR, ^1H NMR, mass spectra, and elemental analysis. Chlorides **16**–**20** were the key intermediates for the synthesis of the final compounds **2**–**13**. Following a procedure described for related compounds,[14] the reaction of 2-amino-4,5-diethoxybenzoic acid with the appropriate cycloketone in the presence of $POCl_3$ afforded **16** and the two isomers **19** and **20**, whereas the reaction between 2-amino-4,5-diethoxybenzoic acid methyl ester and cyclohexanone or cyclopentanone in the presence of P_2O_5 and *N*,*N*-dimethylcyclohexylamine afforded intermediates **14** and **15** that were then transformed into **17** and **18** with $POCl_3$. The reaction between chlorides **16**–**20** and 3-(2-methylthiazol-4-yl)phenylamine[13] gave **2**–**4**, **21**, and **22**. Reduction of **21** and **22** gave **5** and **7**, respectively, that were transformed by acidic hydrolysis into the corresponding amines **6** and **8** (Scheme 1). Although the enantiomers of 3-nitromethylcyclopentanone are known,[15] no attempt was made to synthesize the enantiomers of **5**–**8** because of their significantly lower inhibitory F16BPase activity relative to **2**.

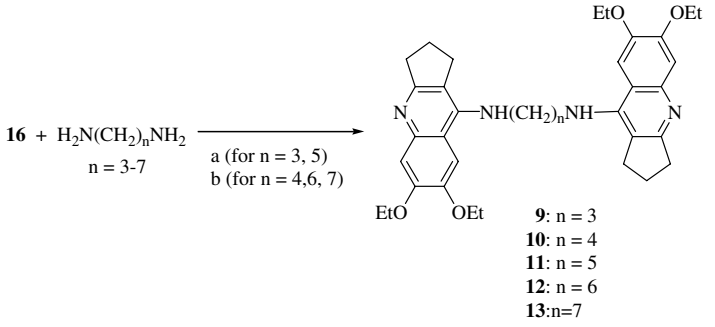
Finally, bis-dihydrocyclopenta[*b*]quinoline derivatives **9**–**13** were obtained through the reaction[16] of chloride **16** and the appropriate 1,ω-alkanediamine (Scheme 2).

2. Results and discussion

Commercial native rabbit liver F16BPase activity was assayed by evaluating the inorganic phosphate hydro-



Scheme 1. Synthesis of quinoline derivatives **2**–**8**. Reagents and conditions: (a) *N*,*N*-dimethylcyclohexylamine, P_2O_5, 170–210 °C; (b) $POCl_3$, reflux; (c) NaI, 1-pentanol, reflux; (d) Raney Ni, MeOH–CH_3COOH, H_2; (e) 12 N HCl.



Scheme 2. Synthesis of dihydrocyclopentaquinoline derivatives **9**–**13**. Reagents and conditions: (a) NaI, 1-pentanol, reflux; (b) phenol, NaI, 170 °C.

lyzed from fructose-1,6-bisphosphate by the enzyme following a described procedure.[13] Thus, compounds **2**–**13** were assayed for their ability to inhibit rabbit F16BPase in comparison with the anilinoquinazoline **1**,[13,17] previously identified as a structurally novel allosteric inhibitor of F16BPase. Since **1** was reported to also have, albeit to a lesser extent, the ability to inhibit EGFR tyrosine kinase activity, **2**–**13** were tested against this target to verify whether they were able to differentiate between F16BPase and EGFR tyrosine kinase activities. Furthermore, since Tacrine dimers were reported to potently inhibit acetylcholinesterase (AChE),[16] **9**–**13** were tested also against this enzyme in comparison with Tacrine, a well-known AChE inhibitor, to assess the selectivity, if any, of these compounds for F16BPase.

The results obtained with **2**–**13** are shown in Table 1 in comparison with those obtained with **1**. An analysis of these results reveals that **2** and its homologs **3** and **4** were potent inhibitors of F16BPase activity, and **2** was even more potent than prototype **1** ($IC_{50} = 2.60 \pm 0.51$ μM vs $IC_{50} = 4.41 \pm 0.07$ μM). However, the replacement of the cyclopentene ring of **2** with a cyclohexene (**3**) or a cycloheptene one (**4**) resulted in a reduction of the F16BPase inhibitory activity, suggesting that the size of the ring is relevant for the interaction. The insertion of a methylacetamide or a methylamine function at position 1 of the cyclopentene ring of **2** caused a dramatic effect on potency as compounds **5** and **6** were devoid of F16BPase inhibitory activity up to 100 μM concentration. On the other hand, when the same substituents were inserted at position 2, the resulting compounds **7** and **8**, albeit significantly less potent than **2**, retained affinity for F16BPase, suggesting that a substituent in this position may be tolerated. The role of the substituents of the cyclopentene ring was investigated by performing a molecular modeling study on **2** by using the crystal structure of complex between F16BPase and a hydroxyl analog of **1**[13] (Fig. 1). In the proposed binding mode for **2**, two inhibitor molecules are harbored into a depthless channel formed by two subunits of F16BPase, and bind to the target in a stacked head-to-tail configuration. The thiazole ring interacts via π–π interaction with the imidazole ring of H55; the oxygen atoms of the two methoxy groups at positions 6 and 7 are able to form H-bonds with the bridging water molecule derived from the origi-

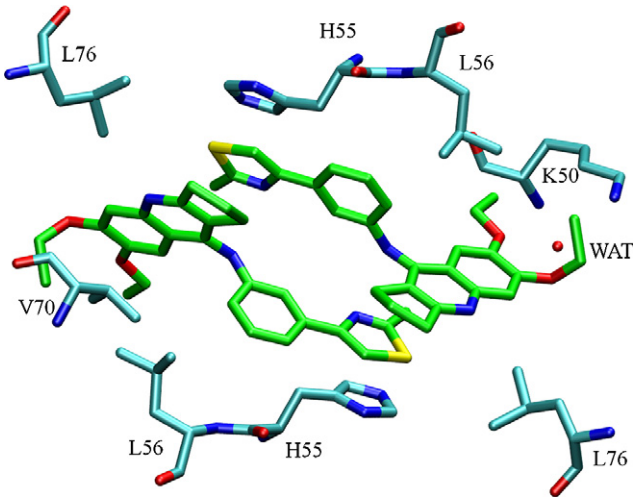


Figure 1. Proposed binding mode of **2** (carbon atoms in green). Two ligand molecules are shown to interact at the F16BPase homotetramer subunit interface (carbon atoms in cyan). Only one bridging water molecule is shown for sake of clarity.

nal crystal structure, in the same way as the hydroxyl analog of **1** does. The dihydrocyclopentaquinoline moiety lies on a lipophilic pocket made by the side chains of L76, L73, V70, L56, I53, and A47. The cyclopentene ring might facilitate both the interactions with this lipophilic pocket so as to enhance the dimeric assembly of the inhibitor. As shown in Figure 1 the position 1 of both cyclopentene rings points against each other with a distance of about 5.5 Å. The presence in this position of a substituent as it is the case of **5** and **6** would interfere with the inhibitor assembly process, thus preventing any interaction between the ligand and the macromolecule. It is not excluded that in a dimeric ligand context the methylthiazolic ring might flip of 180° in order to better interact with the cyclopentene ring of the dimeric counterpart. Furthermore, a bulky function at position 1 of the cyclopentene ring would distort the proper overall shape of the ligand, avoiding any effective interaction with the F16BPase macromolecule. Noteworthy the 2 position of the same ring seems to be much more free to be explored and compatible with some insertions as demonstrated by compounds **7** and **8**. In this context, it would be interesting to investigate whether a substituent at position 3 of the cyclopentene ring of **2** may afford compounds with increased affinity for F16BPase as substituents in this position would not be expected to give rise to any hindrance with the aniline moiety. Unfortunately, we were not able to obtain these kinds of compounds because, in our synthetic procedure, only compounds bearing substituents at positions 1 and 2 were invariably obtained.

Since anilinoquinazolines were reported to discriminate between F16BPase and EGFR tyrosine kinase inhibitory activity, we tested **2** against EGFR tyrosine kinase from A431 cells. It turned out that **2** is a weaker EGFR tyrosine kinase inhibitor than **1** as revealed by their respective IC_{50} values (19.74 ± 1.77 μM vs 11.92 ± 0.95 μM). Clearly, this finding suggests that the dihydro-1*H*-cyclopenta[*b*]quinolin-9-ylamine moiety may represent an alternative to the quinazolin-4-ylamine functionality of **1** in the design of selective F16BPase inhibitors. To verify whether a dihydro-1*H*-cyclopenta[*b*]quinolin-9-ylamine moiety may represent a suitable scaffold for the synthesis of bivalent ligands able to interact with two allosteric binding sites of the homotetramer subunit interface, we prepared compounds **9**–**13**. It turned out that the F16BPase inhibitory activity is dramatically dependent on the length of the spacer connecting the two dihydro-1*H*-cyclopenta[*b*]quinolin-9-ylamine moieties. In fact, only compound **11** was as potent as **2** in inhibiting F16BPase activity, whereas both lower (**9** and **10**) and higher homologs (**12** and **13**) were inactive up to 100 μM concentration with the exception of **10**, which, at this concentration, gave 20% inhibition of F16BPase activity. Interestingly, although inhibiting AChE to some extent, **11** was a much weaker inhibitor of EGFR tyrosine kinase activity than both **1** and **2**, suggesting that it may represent a new lead for the design of bivalent F16BPase inhibitors without significantly affecting EGFR tyrosine kinase activity.

2.1. Experimental section

2.1.1. Chemistry. Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and direct infusion ESI-MS spectra were recorded on Perkin-Elmer 297 and Waters ZQ 4000 apparatus, respectively. HRMS analyses were performed on MAT95XP Finnigan Thermoelectron apparatus. ^{1}H NMR spectra were recorded on Varian VXR 300 instrument. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Although the IR spectral data are not always included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry. The anilinoquinazoline **1** was prepared according to literature method.[13]

2.1.2. 6,7-Diethoxy-1,3,4,10-tetrahydro-2*H*-acridin-9-one (14). A mixture of 4,5-diethoxy-2-aminobenzoic acid methyl ester (2.0 g, 8.36 mmol), *N*,*N*-dimethylcyclohexylamine (2.50 mL, 16.72 mmol), cyclohexanone (1.04 mL, 10.04 mmol), and P_2O_5 (2.38 g, 16.72 mmol) was heated at 170 °C until the exothermic reaction subsided. The resulting mixture was then heated at 210 °C for 4 h under stirring. The mixture was hydrolyzed with water and 2 N NaOH to pH 10 to give a solid that was filtered off and washed with ethanol and ether and, finally, purified by chromatography. Eluting with a step gradient system of CH_2Cl_2/MeOH (9.6:0.4 to 5:5) afforded **14**: 0.30 g, 12% yield; mp 293–296 °C; ^{1}H NMR (DMSO-d_6) δ 1.47 (t, 3H), 1.49 (t, 3H), 1.81–1.88 (m, 4H), 2.61 (t, 2H), 2.78 (t, 2H), 4.15 (q, 4H), 6.87 (s, 1H), 7.57 (s, 1H).

2.1.3. 2,3-Diethoxy-5,6,7,8,9,10-hexahydrocyclohepta[*b*]quinolin-11-one (15). It was synthesized from 4,5-diethoxy-2-aminobenzoic acid methyl ester (2.0 g, 8.36 mmol), *N*,*N*-dimethylcyclohexylamine (2.50 mL, 16.72), cycloheptanone (1.18 mL, 10.03 mmol), and P_2O_5 (2.38 g, 16.72 mmol) following the procedure described for **14**. It was purified by chromatography eluting with CH_2Cl_2–MeOH (9.7:0.3): 0.54 g, 21% yield; ^{1}H NMR (CD_3OD) δ 1.42–1.58 (m, 6H), 1.59–1.71 (m, 2H), 1.72–1.83 (m, 2H), 1.84–1.93 (m, 2H), 2.84–2.98 (m, 4H), 4.09–4.23 (m, 4H), 6.82 (s, 1H), 7.62 (s, 1H).

2.1.4. 9-Chloro-6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinoline (16). A mixture of 4,5-diethoxy-2-aminobenzoic acid (1.5 g, 6.66 mmol), cyclopentanone (0.56 g, 6.66 mmol), and $POCl_3$ (9 mL) was heated to reflux for 2 h. After removal of $POCl_3$, the brown residue was treated with ice and aqueous 30% ammonia to pH 10. The resulting mixture was evaporated under vacuum to give a residue that was purified by chromatography. Eluting with toluene–EtOAc (8:2) gave **16**: 0.10 g, 5% yield; mp 101–103 °C; ^{1}H NMR ($CDCl_3$) δ 1.54 (t, 3H), 1.58 (t, 3H), 2.22 (quintet, 2H), 3.10 (t, 2H), 3.19 (t, 2H), 4.23 (q, 2H), 4.29 (q, 2H), 7.37 (s, 2H).

2.1.5. 9-Chloro-6,7-diethoxy-1,2,3,4-tetrahydroacridine (17). A solution of **14** (0.32 g, 1.11 mmol) in $POCl_3$ (2.0 mL) was heated to reflux for 2 h. After cooling, the resulting solution was made basic (pH 10) by cautious addition of aqueous 15% ammonia to give a solid that was filtered off, washed with water, and then recrystallized from acetone to give **17** in nearly quantitative yield (0.34 g): mp 113–115 °C; ^{1}H NMR ($CDCl_3$) δ 1.56 (t, 3H), 1.58 (t, 3H), 1.94 (qt, 4H), 2.97–3.03 (m, 2H), 3.04–3.12 (m, 2H), 4.25 (q, 2H), 4.28 (q, 2H), 7.31 (s, 1H), 7.38 (s, 1H).

2.1.6. 11-Chloro-2,3-diethoxy-7,8,9,10-tetrahydro-6*H*-cyclohepta[*b*]quinoline (18). It was obtained starting from **15** (0.52 g, 1.71 mmol) following the procedure described for **17**: 0.18 g, 33% yield; mp 145–146 °C; ^{1}H NMR ($CDCl_3$) δ 1.97 (t, 3H), 1.99 (t, 3H), 1.71–1.88 (m, 4H), 1.89–1.98 (m, 2H), 3.15–3.26 (m, 4H), 4.28 (q, 2H), 4.31 (q, 2H), 7.34 (s, 1H), 7.43 (s, 1H).

2.1.7. 9-Chloro-6,7-diethoxy-1-nitromethyl-2,3-dihydro-1*H*-cyclopenta[*b*]quinoline (19) and 9-chloro-6,7-diethoxy-2-nitromethyl-2,3-dihydro-1*H*-cyclopenta[*b*]quinoline (20). A mixture of 3-nitromethylcyclopentanone[14] (1.42 g, 9.9 mmol), 4,5-diethoxy-2-aminobenzoic acid (2.23 g, 9.9 mmol) in $POCl_3$ (13 mL) was heated to reflux for 2 h. After removal of the solvent under vacuum, the residue was treated with ice and then made basic (pH 10) with aqueous 30% ammonia. The resulting solution was extracted with $CHCl_3$ (3 × 10 mL). Removal of the dried solvent gave a mixture of the two isomers **19** and **20** that were separated by chromatography eluting with toluene–EtOAc (8:2).

Compound **20**: first fraction; 0.11 g, 3% yield; mp 164–166 °C; ^{1}H NMR ($CDCl_3$) δ 1.52–1.61 (m, 6H), 2.86–3.07 (m, 2H), 3.32–3.51 (m, 3H), 4.18–4.33 (m, 4H), 4.50–4.54 (m, 2H), 7.35 (s, 2H).

Compound **19**: second fraction; 0.20 g, 6% yield; mp 204–206 °C; ^{1}H NMR ($CDCl_3$) δ 1.57 (t, 3H), 1.59 (t, 3H), 2.18–2.28 (m, 1H), 2.40–2.57 (m, 1H), 3.09–3.39 (m, 2H), 4.12–4.22 (m, 5H), 4.48 (t, 1H), 4.92 (dd, 1H), 7.38 (s, 2H).

2.1.8. (6,7-Diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)[3-(2-methylthiazol-4-yl)phenyl]amine (2). A solution of **16** (0.10 g, 0.343 mmol), 3-(2-methylthiazol-4-yl)phenylamine[13] (0.066 g, 0.343 mmol), and NaI (catalytic amount) in pentanol (3 mL) was heated to reflux for 6 h. After cooling, the solid was filtered and taken up with 1.5 N NaOH to give pure **2** as the free base: 0.089 g, 58% yield; mp 289–291 °C; ^{1}H NMR (CD_3OD) δ 1.48 (t, J = 7.0 Hz, 3H), 1.54 (t, J = 6.7 Hz, 3H), 2.10 (m, 2H), 2.43 (t, J = 7.0 Hz, 2H), 2.75 (s, 3H), 3.14 (t, J = 6.7 Hz, 2H), 4.18 (q, J = 7.0 Hz, 2H), 4.28 (q, J = 7.0 Hz, 2H), 7.17 (s, 1H), 7.22 (d, J = 6.8 Hz, 1H), 7.51 (t, J = 7.6 Hz, 1H), 7.57 (s, 1H), 7.63 (s, 1H), 7.67 (s, 1H), 7.85 (d, J = 7.6 Hz, 1H); MS (ESI$^+$): *m*/*z* 446 (M+H)$^+$; HRMS *m*/*z* calcd for $C_{26}H_{27}N_3O_2S$ 445.18240, found 445.18153.

2.1.9. (6,7-Diethoxy-1,2,3,4-tetrahydroacridin-9-yl)[3-(2-methylthiazol-4-yl)phenyl]amine (3). It was obtained starting from **17** (0.34 g, 1.11 mmol) and 3-(2-methylthiazol-4-yl)phenylamine[13] (0.21 g, 1.11 mmol) following the procedure described for **2**: 0.48 g, 94% yield; mp 249–251 °C; ^{1}H NMR (CD_3OD) δ 1.27 (t, 3H), 1.54 (t, 3H), 1.84–2.04 (m, 4H), 2.63–2.72 (m, 2H), 2.76 (s, 3H), 3.09 (t, 2H), 3.77 (q, 2H), 4.26 (q, 2H), 7.05-7.19 (m, 2H), 7.50 (t, 1H), 7.66–7.80 (m, 4H); MS (ESI$^+$): *m*/*z* 460 (M+H)$^+$; HRMS *m*/*z* calcd for $C_{27}H_{29}N_3O_2S$ 459.19750, found 459.19739.

2.1.10. (2,3-Diethoxy-7,8,9,10-tetrahydro-6*H*-cyclohepta[*b*]quinolin-11-yl)[3-(2-methylthiazol-4-yl)phenyl]amine (4). It was obtained starting from **18** (0.15 g, 0.47 mmol) and 3-(2-methylthiazol-4-yl)phenylamine[13] (0.09 g, 0.47 mmol) following the procedure described for **2**: 0.044 g, 20% yield; mp 224–226 °C; ^{1}H NMR (CD_3OD) δ 1.35 (t, 3H), 1.52 (t, 3H), 1.61–1.72 (m, 2H), 1.78–1.97 (m, 4H), 2.73 (s, 3H), 2.93–3.05 (m, 2H), 3.16–3.24 (m, 2H), 4.00 (q, 2H), 4.24 (q, 2H), 6.58 (d, 1H), 7.15–7.36 (m, 5H), 7.47 (s, 1H); MS (ESI$^+$): *m*/*z* 474 (M+H)$^+$; HRMS *m*/*z* calcd for $C_{28}H_{31}N_3O_2S$ 473.21370, found 473.21341.

2.1.11. (6,7-Diethoxy-1-nitromethyl-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)[3-(2-methylthiazol-4-yl)phenyl]-amine (21). A solution of **19** (0.20 g, 0.57 mmol), 3-(2-methylthiazol-4-yl)phenylamine[13] (0.11 g, 0.57 mmol), and NaI (catalytic amount) in pentanol (4 mL) was heated to reflux for 6 h. After cooling the resulting solution, the solid was filtered and washed with petroleum ether to give pure **21**: 0.205 g, 71% yield; mp 269–271 °C; ^{1}H NMR (CD_3OD) δ 1.45 (t, 3H), 1.54 (t, 3H), 2.09–2.21 (m, 1H), 2.38–2.53 (m, 1H), 2.74 (s, 3H), 3.16–3.39 (m, 1H), 3.63–3.73 (m, 1H), 2.66–2.76 (m, 2H), 4.29 (q, 4H), 4.41 (dd, 1H), 7.21 (s, 1H), 7.33 (s, 1H), 7.55 (t, 1H), 7.74 (d, 2H), 7.86 (d, 2H).

2.1.12. *N*-(6,7-Diethoxy-9-[3-(2-methylthiazol-4-yl)phenylamino]-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-1-yl)-acetamide (5). A suspension of **21** (0.205 g, 0.405 mmol) and Raney Ni (nickel sponge, suspension in water) (0.10 g) in MeOH (30 mL) and CH_3COOH (5 mL) was hydrogenated at room temperature until the theoretical amount of hydrogen was consumed. Following catalyst removal, the solvent was evaporated, yielding a residue that was purified by chromatography. Eluting with $CHCl_3$–MeOH–aqueous 30% ammonia (48:2:0.04) gave **7**: 0.075 g, 36% yield; mp 120–122 °C; ^{1}H NMR (CD_3OD) δ 1.32 (t, 3H), 1.53 (t, 3H), 1.80 (s, 3H), 1.98–2.42 (m, 2H), 2.85 (s, 3H), 2.91–3.24 (m, 4H), 3.38–3.58 (m, 2H), 3.97 (q, 2H), 4.22 (q, 2H), 6.89 (d, 1H), 7.23–7.56 (m, 6H); MS (ESI$^+$): *m*/*z* 517 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{29}H_{32}N_4O_3S$ 516.21951, found 516.21890.

2.1.13. (1-Aminomethyl-6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)[3-(2-methylthiazol-4-yl)phenyl]-amine (6). A solution of **5** (0.03 g, 0.058 mmol) in 12 N HCl (4 mL) was heated overnight at reflux. After cooling, the solution was made basic with aqueous 35% NaOH and then extracted with $CHCl_3$ (3×5 mL). Removal of dried solvents gave a residue that was purified by chromatography. Eluting with $CHCl_3$–MeOH–aqueous 30% ammonia (90:10:0.05) gave pure **6**: 0.011 g, 39% yield; mp 109–111 °C; ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H), 1.55 (t, 3H), 1.77–1.91 (m, 1H), 2.05 (broad s, exchangeable with D_2O, 2H), 2.30–2.44 (m, 1H), 2.77 (s, 3H), 2.88 (t, 1H), 2.97–3.14 (m, 1H), 3.17–3.30 (m, 2H), 3.38–3.49 (m, 1H), 3.71–3.92 (m, 2H), 4.26 (q, 2H), 6.64 (d, 1H), 7.01 (s, 1H), 7.20 (t, 2H), 7.34–7.42 (m, 3H); MS (ESI$^+$): *m*/*z* 475 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{27}H_{30}N_4O_2S$ 474.20895, found 474.20815.

2.1.14. (6,7-Diethoxy-2-nitromethyl-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)[3-(2-methylthiazol-4-yl)phenyl]-amine (22). It was synthesized starting from **20** (0.11 g, 0.313 mmol) and 3-(2-methylthiazol-4-yl)phenylamine[13] (0.06 g, 0.313 mmol) following the procedure described for **2**: 0.09 g, 56% yield; mp 211–213 °C; ^{1}H NMR (CD_3OD) δ 1.47 (t, 3H), 1.58 (t, 3H), 2.29–2.45 (m, 1H), 2.62–2.75 (m, 1H), 2.78 (s, 3H), 3.04–3.30 (m, 2H), 4.21 (q, 2H), 4.31 (q, 2H), 4.57 (d, 2H), 7.18 (s, 1H), 7.19 (d, 2H), 7.57 (t, 1H), 7.78 (d, 1H), 7.81 (s, 1H), 7.90 (d, 1H).

2.1.15. *N*-(6,7-Diethoxy-9-[3-(2-methylthiazol-4-yl)phenylamino]-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-2-yl)acetamide (7). It was synthesized starting from **22** (0.09 g, 0.18 mmol) following the procedure described for **5**: 0.032 g, 34% yield; mp 113–115 °C; ^{1}H NMR (CD_3OD) δ 1.25–1.40 (m, 1H), 1.38 (t, 3H), 1.51 (t, 3H), 1.89 (s, 3H), 2.29–2.44 (m, 1H), 2.50–2.92 (m, 3H), 2.71 (s, 3H), 3.07–3.27 (m, 3H), 4.10 (q, 2H), 4.22 (q, 2H), 6.90 (d, 1H), 7.21–7.64 (m, 6H); MS (ESI$^+$): *m*/*z* 517 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{29}H_{32}N_4O_3S$ 516.21951, found 516.21910.

2.1.16. (2-Aminomethyl-6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)[3-(2-methylthiazol-4-yl)phenyl]-amine (8). It was synthesized starting from **7** (0.029 g, 0.056 mmol) following the procedure described for **6**: 0.008 g, 30% yield; mp 169–171 °C; ^{1}H NMR ($CDCl_3$) δ 1.42 (t, 3H), 1.54 (t, 3H), 2.22 (broad s, exchangeable with D_2O, 1H), 2.40–2.88 (m, 6H), 2.76 (s, 3H), 3.18–3.38 (m, 1H), 4.02 (q, 2H), 4.22 (q, 2H), 6.42 (broad s, exchangeable with D_2O, 2H), 6.74 (d, 1H), 7.04–7.48 (m, 6H); MS (ESI$^+$): *m*/*z* 475 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{27}H_{30}N_4O_2S$ 474.20895, found 474.20810.

2.1.17. *N*,*N*′-Bis-(6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)propan-1,3-diamine (9). Propan-1,3-diamine (0.0278 g, 0.375 mmol) and NaI (catalytic amount) were added to a solution of **16** (0.22 g, 0.75 mmol) in pentanol (5 mL). After refluxing for 32 h under a stream of nitrogen, the solvent was removed under vacuum to give a residue that was purified by chromatography. Eluting with a step gradient system of CH_2Cl_2–MeOH (9.8:0.2 to 9.5:0.5) afforded a solid that was taken up in 1 N NaOH, the resulting mixture was stirred for 1 h to give **9** as free base: 0.03 g, 14% yield; mp 240–242 °C; ^{1}H NMR (DMSO-d_6) δ 2.98 (t, 4H), 3.15 (t, 4H), 3.78–3.92 (m, 4H), 4.02 (q, 4H), 4.10 (q, 4H), 7.0 (s, 2H), 7.52 (s, 2H); MS (ESI$^+$): *m*/*z* 585 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{35}H_{44}N_4O_4$ 584.33626, found 584.33533.

2.1.18. *N*,*N*′-Bis-(6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)butan-1,4-diamine (10). A mixture of butan-1,4-diamine (6.7 mg, 0.754 mmol), **16** (0.44 g, 1.508 mmol), phenol (1.5 g), and KI (catalytic amount) was heated for 2 h at 170 °C under a stream of dry nitrogen to afford a residue that was taken up with EtOAc. The resulting mixture was washed with aqueous 10% NaOH to afford a solid that was purified by chromatography (step gradient system: CH_2Cl_2–MeOH–aqueous 23% ammonia, 9.8:0.2:0.0 to 9.5:0.5:0.02): 0.055 g, 12% yield; mp 158–160 °C (dec); ^{1}H NMR (CD_3OD) δ 1.52 (t, 6H), 1.59 (t, 6H), 1.80–1.98 (m, 4H), 2.00–2.15 (m, 4H), 2.88–3.08 (m, 8H), 3.60–3.91 (complex m, 4H), 4.20 (q, 8H), 6.98 (s, 2H), 7.42 (s, 2H); MS (ESI$^+$): *m*/*z* 599 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{36}H_{46}N_4O_4$ 598.35191, found 598.35203.

2.1.19. *N*,*N*′-Bis-(6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)pentan-1,5-diamine (11). It was obtained starting from **16** (0.22 g, 0.75 mmol) and pentan-1,5-diamine (0.0383 g, 0.375 mmol) as described for **9**: 0.017 g, 10% yield; mp 170–173 °C (dec); ^{1}H NMR ($CDCl_3$) δ 1.51 (t, J = 7.0 Hz, 6H), 1.57 (t, J = 6.8 Hz, 6H), 1.65–1.90 (m, 6H), 2.08–2.17 (m, 4H), 2.98–3.18 (complex m, 8H), 3.52–3.63 (m, 4H), 4.08 (q, J = 6.8 Hz, 4H), 4.12 (q, J = 7.0 Hz, 4H), 7.03 (s, 2H), 7.32 (s, 2H); MS (ESI$^+$): *m*/*z* 613 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{37}H_{48}N_4O_4$ 612.36756, found 612.36664.

2.1.20. *N*,*N*′-Bis-(6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)hexan-1,6-diamine (12). It was obtained from hexan-1,6-diamine (8.8 mg, 0.754 mmol) and **16** (0.44 g, 1.508 mmol) as described for **10**: 0.208 g, 40% yield; mp 148–150 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.35 (t, 6H), 1.43 (t, 6H), 1.48–1.63 (complex m, 4H), 1.98–2.12 (m, 4H), 2.15–2.38 (m, 4H), 2.81–2.98 (m, 4H), 3.01–3.20 (m, 4H),3.42–3.58 (m, 4H), 4.12 (q, 8H), 7.10 (s, 2H), 7.56 (s, 2H); MS (ESI$^+$): *m*/*z* 627

$(M+H)^+$; HRMS *m/z* calcd for $C_{38}H_{50}N_4O_4$ 626.38321, found 626.38341.

2.1.21. *N,N′*-Bis-(6,7-diethoxy-2,3-dihydro-1*H*-cyclopenta[*b*]quinolin-9-yl)heptan-1,7-diamine (13). It was obtained from heptan-1,7-diamine (9.8 mg, 0.754 mmol) and **16** (0.44 g, 1.508 mmol) as described for **10**: 0.082 g, 15% yield; mp 102–105 °C; ^1H NMR ($CDCl_3$) δ 1.24–1.38 (m, 4H), 1.43 (t, 6H), 1.57 (t, 6H), 1.61–1.78 (m, 4H), 1.97–2.24 (complex m, 6H), 2.95–3.18 (m, 8H), 3.43–3.70 (m, 4H), 4.05–4.35 (dd, 8H), 7.18 (s, 2H), 7.29 (s, 2H); MS (ESI^+): *m/z* 641 $(M+H)^+$; HRMS *m/z* calcd for $C_{39}H_{52}N_4O_4$ 640.39886, found 640.39904.

2.2. Biology

2.2.1. EGFR tyrosine kinase assay. The epidermal growth factor receptor (EGFR) was isolated from A431 cells by immunoprecipitation as previously reported.[18] The inhibition of the compounds on EGFR tyrosine kinase activity was determined using Non-radioactive Tyrosine Kinase Activity Assay Kit (Chemicon® International, Temecula, USA) in accordance with the manufacturer's instructions. Briefly, the kinase reaction was initiated by adding ATP/$MgCl_2$ solution to assay mixture containing biotinylated tyrosine kinase substrate poly(Glu/Tyr) 4:1, immunoprecipitated EGFR, and test compound (0.2–200 μM). After 60 min at 30 °C, the reaction was stopped with 120 mM EDTA. Fifty microliters of the reaction mixture was transferred in streptavidin-coated strip wells and incubated for 30 min at 37 °C. The wells were then washed with wash buffer, and phosphotyrosine specific monoclonal antibody conjugated to horseradish peroxidase was added to wells for 1 h. After washing the wells twice with wash buffer, tetramethylbenzidine substrate solution was added to each well. The amount of colored product was measured (450 nm) with spectrophotometer (Spectra model Classic, TECAN®, Maennedorf, Switzerland). At least two independent dose–response curves were obtained and the concentration of compound resulting in 50% inhibition of tyrosine kinase activity (IC_{50}) calculated.

2.2.2. Fructose-1,6-bisphosphatase enzyme assay. Purified native fructose-1,6-bisphosphatase (F16BPase) from rabbit liver (EC 3.1.3.11) was obtained from Sigma-Aldrich (Milan, Italy). F16BPase (9 ng/μL) and substrate (625 μM, fructose-1,6-bisphosphate) solutions were prepared in 50 mM Hepes/NaOH buffer (pH 7.2), containing 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, and 1 mM DTT. Stock solutions of the tested compounds were prepared in methanol at 0.2–310 μM concentration range and diluted with Hepes buffer. Ammonium molibdate/malachite green (AM/MG) solution was prepared by mixing one volume of 4.2% ammonium molibdate (w/v) in 4 M hydrochloric acid with three volumes of 0.045% malachite green (w/v) aqueous solution containing 0.01% Tween 20 (v/v). The resulting mixture was stirred at room temperature for 30 min and filtered through 0.22 μm filter before use. F16BPase activity was assayed by measuring its ability to hydrolyze inorganic phosphate from fructose-1,6-bisphosphate using a modification of a previously reported method.[13,17] Enzyme activity was assayed spectrophotometrically, determining the phosphate released in the enzymatic assay buffer under saturating substrate concentrations. Briefly, 60 μL of rabbit liver F16BPase (0.54 μg) was incubated with 240 μL of substrate (final saturating concentration 500 μM) in a 1 mL plastic cuvette for 20 min at 37 °C. Phosphate released by the enzymatic reaction was transformed into a colored complex showing a maximum absorption at 620 nm, by the addition of 700 μL AM/MG solution to the reaction cuvette. After 20 min, spectrophotometric assay was performed by reading absorbance at 620 nm with a blank containing all components except F16BPase in order to account for nonenzymatic reaction. F16BPase maximum activity was expressed as $\Delta A\ min^{-1}$ at 620 nm. Test compounds were added to the assay solution containing F16BPase before the addition of the substrate. Different concentrations of each compound in assay solution were used in order to obtain inhibition of F16BPase activity ranging between 20 and 80%. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in duplicate. The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (v_i/v_0 \times 100)$, where v_i is the initial rate calculated in the presence of inhibitor and v_0 is the enzyme activity. Inhibition curves were obtained for each compound by plotting the % inhibition versus the logarithm of inhibitor concentration in the assay solution. The linear regression parameters were determined for each curve and the IC_{50} extrapolated.

2.2.3. Inhibition of AChE. The method of Ellman et al.[19] was followed. The assay solution consisted of a 0.1 M phosphate buffer, pH 8.0, with the addition of 340 μM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.02 U/mL of human recombinant AChE (Sigma Chemical), and 550 μM of substrate (acetylthiocholine iodide). Test compounds were added to the assay solution and preincubated at 37 °C with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE in order to account for non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values, when possible, were determined graphically from log concentration–inhibition curves.

2.2.4. Molecular modeling. The dimeric molecular structure (pdb code: 1kz8), and the tetrameric biological unit coordinates, of the F16BPase in complex with the hydroxyl analog of **1**, {4-[3-(6,7-diethoxy-quinazolin-4-ylamino)-phenyl]-thiazol-2-yl}-methanol, was obtained from the Protein Data Bank.[20] A three-dimensional model of **2** was generated by properly modifying the soaked ligand by means of the SYBYL 7.1 molecular modeling suite (Tripos Inc., St. Louis, MO) and then optimizing the geometry at the B3LYP/6-31G* level of theory using the Gussian03 software (Gaussian, Inc.

Wallingford, CT). DFT calculations were carried out at the supercomputer center CINECA (Casalecchio sul Reno, Italy). Compound **2** was docked into the same allosteric binding site of the soaked **1** analog using as target macromolecule the dimeric form of F16BPase; all water molecules were deleted but number 108 was maintained because of its bridging role between Lys50 side chain, and the oxygen atoms of the ortho-diethoxy groups of **1** analog. The GOLD software[21] (v 3.0.1) was used to perform the docking calculation. The binding site was formed by the γC atom of Leu56 and every residues within 15 Å. In any case the *detect cavity* option was selected. The water molecule was taken fixed but its hydrogen atoms free to move in order to optimize H-bond interactions. The *default settings* parameters were set for the Genetic Algorithm and the ChemScore[22] used to drive and rank the docking poses. The binding mode of **1** analog was taken as reference and because of the exposed character of the allosteric binding site, docking poses poorly reproduced (about 5%) the binding mode of the original ligand. In order to better sample the interested configurational space, a soft template similarity constraint was added using as template the relative coordinates of the **1** analog and a constraint weight of 10,00. The best ranked docking solution of **2** was selected and used to generate, for symmetry with the tetrameric biological unit coordinates, the potential two crystal-symmetry related molecules of the inhibitor bound to the homotetramer subunit interface as reported in the Results and Discussion Section.

Acknowledgments

This work was supported by grants from MIUR, Rome, and the Alma Mater Studiorum – University of Bologna.

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