Bioorganic & Medicinal Chemistry 17 (2009) 2658–2672

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis, activity and molecular modeling of a new series of chromones as low molecular weight protein tyrosine phosphatase inhibitors

Marco Forghieri [a], Christian Laggner [b], Paolo Paoli [c], Thierry Langer [d], Giampaolo Manao [c], Guido Camici [e], Lucia Bondioli [f], Fabio Prati [a], Luca Costantino [f,*]

[a] Department of Chemistry, University of Modena and R.E., Via G. Campi 183, 41100 Modena, Italy
[b] Department of Pharmaceutical Chemistry, Institute of Pharmacy and Center for Molecular Biosciences (CMBI), University of Innsbruck, Innrain 52c, A-6020 Innsbruck, Austria
[c] Department of Biochemical Sciences, University of Florence, Viale Morgagni 50, 50134 Florence, Italy
[d] Prestwick Chemical Inc., Bld. Gonthier d'Andernach, 67400 Strasbourg-Illkirch, France
[e] Center of Excellence for Scientific Research DENOThe, Viale Morgagni 44, 50134 Florence, Italy
[f] Department of Pharmaceutical Sciences, University of Modena and R.E., Via G. Campi 183, 41100 Modena, Italy

ARTICLE INFO

Article history:
Received 3 January 2009
Revised 25 February 2009
Accepted 28 February 2009
Available online 5 March 2009

Keywords:
Low molecular weight protein tyrosine phosphatases
Protein tyrosine phosphatase 1B
Diabetes
Chromones
Antitumoral drugs

ABSTRACT

Protein tyrosine phosphatases (PTP) are crucial elements in eukaryotic signal transduction. Several reports suggested that the LMW-PTP family has oncogenic relevance. Moreover, LMW-PTP has been recognized as a negative regulator of insulin-mediated mitotic and metabolic signaling. Thus, inhibition of the LMW-PTP can be considered an attractive approach for the design of new therapeutic agents for the treatment of type II diabetes and for new antitumoral drugs. To date very few (and weak) inhibitors of LMW-PTP have been identified. On the basis of the reported weak activity of some flavonoids on phosphatases, we discovered a lead that originated a new class of highly active LMW-PTP inhibitors; these compounds inhibit also PTP-1B and are active in cellular assays. Docking experiments and SAR highlighted the possible binding mode of these compounds to the enzyme, putting the background for the future optimization of their inhibitory activity and selectivity towards the closely related enzyme PTP-1B.

1. Introduction

Tyrosine phosphorylation and dephosphorylation are crucial elements in eukaryotic signal transduction.[1–3] One major group of enzymes responsible for hydrolyzing the phosphotyrosyl residues of protein substrates is that of protein tyrosine phosphatases (PTPs).

The PTP superfamily is composed by at least three families:[4,5] (i) 'classical PTPs', which exist both as transmembrane forms (such as LAR or PTP-α) and non transmembrane forms (such as PTP-1B or TC-PTP). They share at least one conserved catalytic domain of 240 residues and are phosphotyrosine specific; (ii) the dual-specificity phosphatases, which are able to dephosphorylate both phosphotyrosine and phosphothreonine in specific sequence contexts; (iii) the low molecular weight phosphotyrosine protein phosphatases (LMW-PTPs) that have a molecular mass of 18 kDa, and are phosphotyrosine specific. A minimal signature sequence for the phosphate binding site, CXXXXXR, is highly conserved among all the PTPs.[6] They share a common catalytic mechanism: in the catalysis pathway, the cysteinyl residue of the signature sequence executes a nucleophilic attack upon the phosphate moiety of the substrate, leading to the formation of a thio-phosphate intermediate. Two isoenzymes of the LMW-PTP, HPTP-A and HPTP-B, corresponding to the fast and the slow electrophoretic forms, are expressed in humans. They are produced by an alternative splicing mechanism, and thus display identical sequences except for residues 40–73.[7] Couples of LMW-PTP isoenzymes have been isolated from other vertebrate sources, termed isoform-1 (IF1) and isoform-2 (IF2), respectively. The differing amino acid sequence as well as the substrate specificity and the differing sensitivity to inhibitors suggest that the two isoenzymes have different function in the cell.

Several reports in the literature have suggested that the LMW-PTP family has oncogenic relevance. In particular, Chiarugi et al. reported that the LMW-PTP oncogenic potential is mediated by its EphA2 tyrosine dephosphorylating activity and that dephosphorylated EphA2 overexpression is highly associated with most human cancers.[8] The overexpression of LMW-PTP is sufficient to transform epithelial cells.[9] High levels of LMW-PTP has been detected in some types of human tumors.[9,10] Moreover, LMW-PTP has been recognized as a negative regulator of insulin-mediated mitotic and metabolic signalling.[11,12] Thus, inhibition of the LMW-PTP can be considered an attractive approach for the design of new

* Corresponding author. Tel.: +39 059 2055125; fax: +39 059 2055131.
E-mail address: luca.costantino@unimore.it (L. Costantino).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.02.060

therapeutic agents for the treatment of type II diabetes and for new antitumoral drugs.

Another PTP, PTP-1B, is involved in diabetes. This phosphatase negatively regulates insulin receptor and insulin receptor substrate-1 phosphorylation. Mice that lack the PTP-1B gene have increased insulin sensitivity with resistance to weight gain on a high fat diet and are otherwise normal. This enzyme is object of an intense search for new compounds able to treat type II diabetes and obesity.[13]

LMW-PTP possesses a phosphate binding loop that has both a similar amino acid sequence (CLGNICR vs CSAGIGR) and a virtually identical backbone structure as that of PTP-1B. Apart from this short sequence, no sequence homology between high and low molecular weight PTPs has been found. The rest of the two binding sites is build up by different structural elements, although both binding sites share one aspartic acid and one tyrosine (or tryptophane in the case of LMP-PTP isoform II) residue at similar positions. Figure 1 shows an overlay of human LMW-PTP isoform I with human PTP-1B (PDB structures 5PNT[14] and 1G7G[15]). A short discussion about binding site similarities is given in Ref. 14.

To date very few (and weak) inhibitors of LMW-PTP have been found; among these, a series of thiazolidinediones were shown to be able to inhibit LMW-PTP besides their primary target PTP-1B.[16] Moreover, a virtual screening method selected, from a database of almost 500,000 commercially available compounds, a subset of 34 compounds; nine of these were experimentally confirmed to be competitive inhibitors of bovine LMW-PTP (this enzyme shares a 94% identity with human LMW-PTP-B), two of them showing K_i values around 10 μM.[17] No data were reported for PTP-1B inhibition exerted by these compounds. Other inhibitors with IC_{50} values in the millimolar range include flavones,[18] the buffer constituent HEPES[19] and phosphonic acids.[20] The flavonoids were tested on the bovine kidney LMW-PTP, and the results obtained highlighted the importance of the position of the hydroxy groups in the structure for their activity—in particular the hydroxy groups

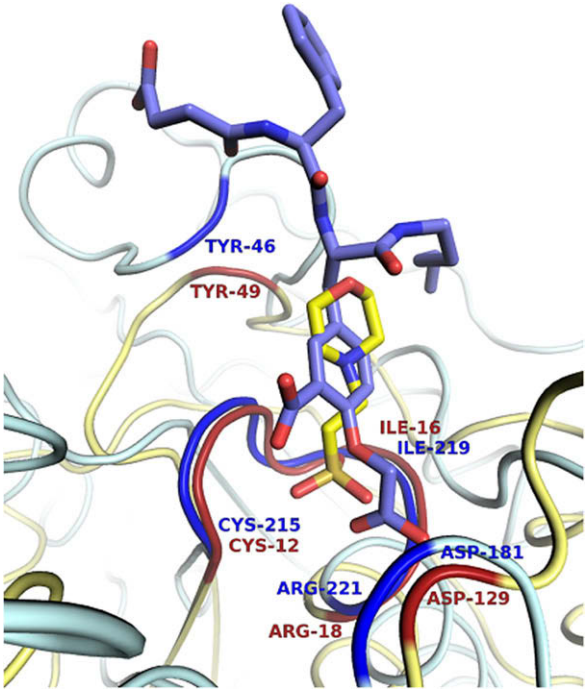


Figure 1. Overlay of ligand binding sites of human LMW-PTP isoform 1 (PDB code 5PNT; yellow and red) and human PTP-1B (1G7G; cyan, blue and purple). The phosphate binding loop and the conserved Tyr and Asp residues are marked in red and blue, respectively. Conserved residues are labeled.

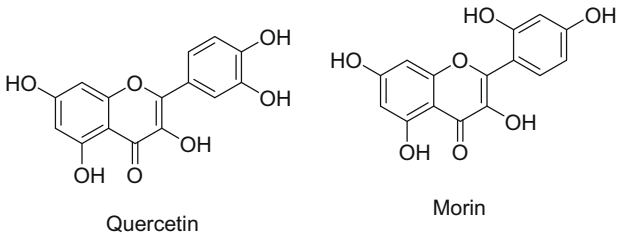


Chart 1. Structures of quercetin and morin.

at positions C3′ and C4′ (quercetin) or on C2′ and C4′ (morin) of the 2-phenyl residue (Chart 1).[18] While the flavonoids were tested in a screening program, phosphonic acids were designed on the basis of the activation of HCPTP-B and inhibition of HCPTP-A exerted by adenine.[20]

It is also known that flavonoids are inhibitors of cyclic AMP phosphodiesterase; SAR studies together with the observed competitive kinetics for cAMP suggests that flavonoids compete with cAMP for a nucleotide binding site at which stacking occurs.[21] Quantum chemical studies showed that the charge distribution of the pyrone ring of flavone resembles that of the pyrimidine ring of the adenine present in cAMP.[21] Both ring systems are planar, aromatic, and of similar size. Furthermore, we found that 3-hydroxy-flavones and adenine residues often share similar positions and hydrogen bonding patterns in certain kinases (phosphoinositide 3-kinase, CDK2, and Pim-1; compare PDB structures 1E8W with 1E8X, 2DUV with 1HCK, and 2O3P with 1YI4), with the 3-hydroxy and 4-carbonyl oxygen atoms of the flavones aligning with the 6-amino nitrogen atom and the ring nitrogen atom at position 1 of the adenine structures, respectively.

Among the tested flavonoids, quercetin behaves as an activator of the enzyme. It is yet unclear whether the mechanism is similar to that proposed by Wang for the activation of LMW-PTP by adenine, where a water molecule participates at the active site to improve catalysis.[22] Furthermore, quercetin and related compounds have been reported to inhibit the closely related enzyme PTP-1B (IC_{50} = 23.3 μM).[23]

On the basis of these findings, we decided to screen several in-house flavonoids with in vitro enzyme inhibition assays against both LMW-PTP isoforms in order to detect a lead for further optimization. This led to the discovery of compound **1** (Table 1) which in turn led to the preparation and testing of a small initial series of compounds (**2–8**, Table 1).

Docking studies were then conducted in order to understand the binding mode of these compounds. On the basis of the initial results obtained, two series of compounds (Chart 2, compounds **9–15** and **18–20** of general formula **A**, (Tables 1 and 2) and **16, 17**, general formula **B**, Table 1) were prepared and their inhibitory activity against the two LMW-PTP isoforms assessed. The results from the binding assays were then used to reevaluate the docking results in order to gain insight into their possible binding modes, allowing the development of more active compounds in the future.

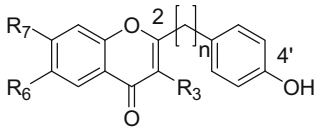
Moreover, the activity of representative compounds of this class were tested for their ability to inhibit the phosphatases in cells, and thus activate the insulin signaling pathway.

2. Results and discussion

2.1. Chemistry

The substituted 4*H*-1-benzopyran-4-one derivatives **1,5–7** were synthetized from the appropriate 4-substituted-2′-hydroxy-4′-phenyldihydrochalchones **23a–b** by reaction with the appropriate ester in the presence of NaH/pyridine (Scheme 1), following the

Table 1
Structure and enzyme inhibition data of compounds **1**–**17**



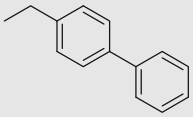
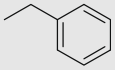
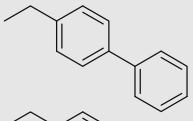
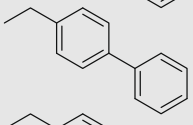
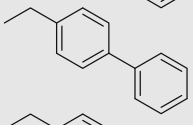
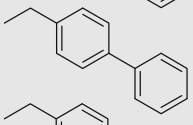
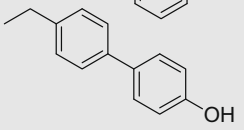
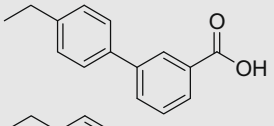
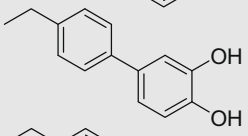
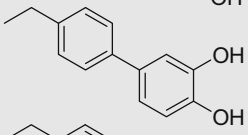
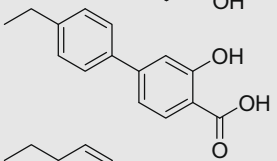
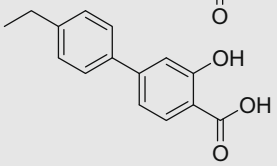
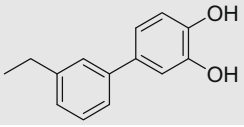
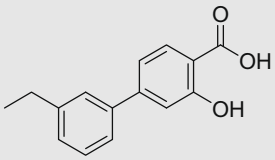
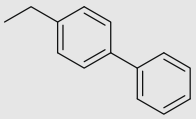
Compound	R_6	R_7	R_3	*n*	IF I, μM (SD)[a]	IF II, μM (SD)[a]	PTP-1B, μM (SD)[a]
1	H	OH		0	5.7 (0.1)	13.9 (0.4)	4% inh. at 15 μM
2	H	OH	H	1	10% inh. at 15 μM	10% inh. at 15 μM	0% inh. at 15 μM
3	H	OH		1	10% inh. at 15 μM	10% inh. at 15 μM	0% inh. at 15 μM
4	H	OH		1	5.3 (0.2)	17.5 (0.5)	10% inh. at 15 μM
5	H	OH		2	6.5 (0.2)	17.3 (0.4)	20% inh. at 15 μM
6	H	OH		3	10% inh. at 15 μM	10% inh. at 15 μM	0% inh. at 15 μM
7	OH	H		1	8.6 (0.4)	17.2 (0.4)	20% inh. at 15 μM
8	H	OH		0	8.2 (0.4)	18.4 (0.1)	17.0 (0.1)
9	H	OH		0	11.4 (0.1)	26.8 (0.6)	6.2 (0.1)
10	H	OH		0	7.4 (0.1)	12.4 (0.1)	7.7 (0.1)
11	H	H		0	14.3 (0.4)	12.6 (0.1)	5.0 (0.2)
12	H	OH		0	1.3 (0.02)	2.2 (0.1)	1.0 (0.3)
13	H	H		0	0.7 (0.05)	5.7 (0.04)	0.9 (0.04)
14	H	OH		0	60% inh. at 20 μM	25% inh. at 20 μM	50% inh. at 20 μM

Table 1 (*continued*)

Compound	R_6	R_7	R_3	*n*	IF I, μM (SD)[a]	IF II, μM (SD)[a]	PTP-1B, μM (SD)[a]
15	H	OH		0	5 % inh. at 20 μM	10% inh. at 20 μM	50% inh. at 20 μM
16	OH	OH		0	1.1 (0.10)	3.0 (0.06)	2.0 (0.08)
17	OH	OH	H	0	25% inh. at 20 μM	10% inh. at 20 μM	10% inh. at 20 μM

[a] The enzyme inhibition data are expressed as IC_{50} values (SD, Standard deviation) or % inhibition at a given concentration.

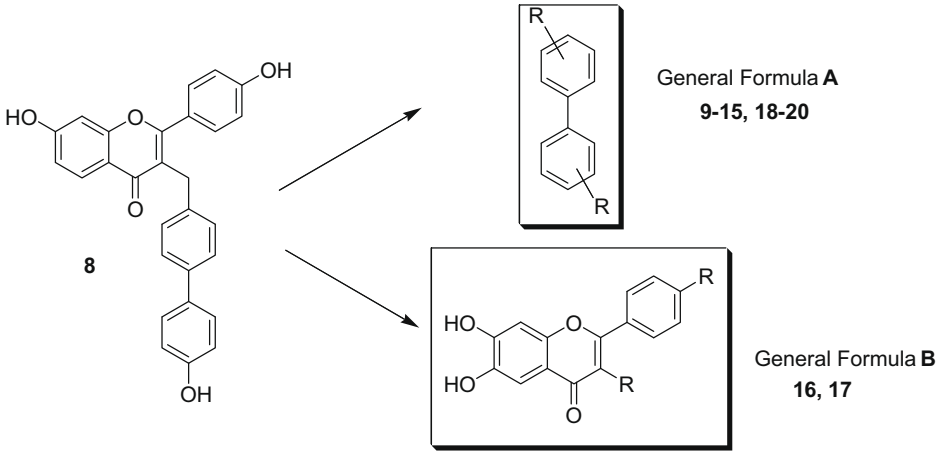


Chart 2. Development of compounds of general formula **A** and **B**.

general procedure described in literature.[24] The intermediate 1,3-diketones were then cyclized with acetic acid/sulphuric acid, to obtain **24a–d**. Then the compounds were deprotected to give **1,5–7** by treatment with BBr_3. Owing to the very low yield obtained in the presence of the dihydrochalchone **23e** following Route A, a different synthetic procedure (Route B) was followed for compounds **8–16**. The dihydrochalchones **23c–f** were acylated in the presence of trietylamine and dimetylaminopyridine (**25a–d**). Claisen condensation with NaH in DMSO at rt and subsequent cyclization with acetic acid/sulphuric acid afforded the corresponding compounds **27a–d**. The bromo derivatives **27b–d** were subjected to Suzuki coupling with (substituted)phenylboronic acids in order to obtain the biphenyl derivatives **28a–h**. The fully protected derivatives were deprotected with the aid of BBr_3 to obtain **8–16**. Suzuki coupling, followed by BBr_3 deprotection was also applied for the synthesis of compounds **18–20** (Scheme 2).

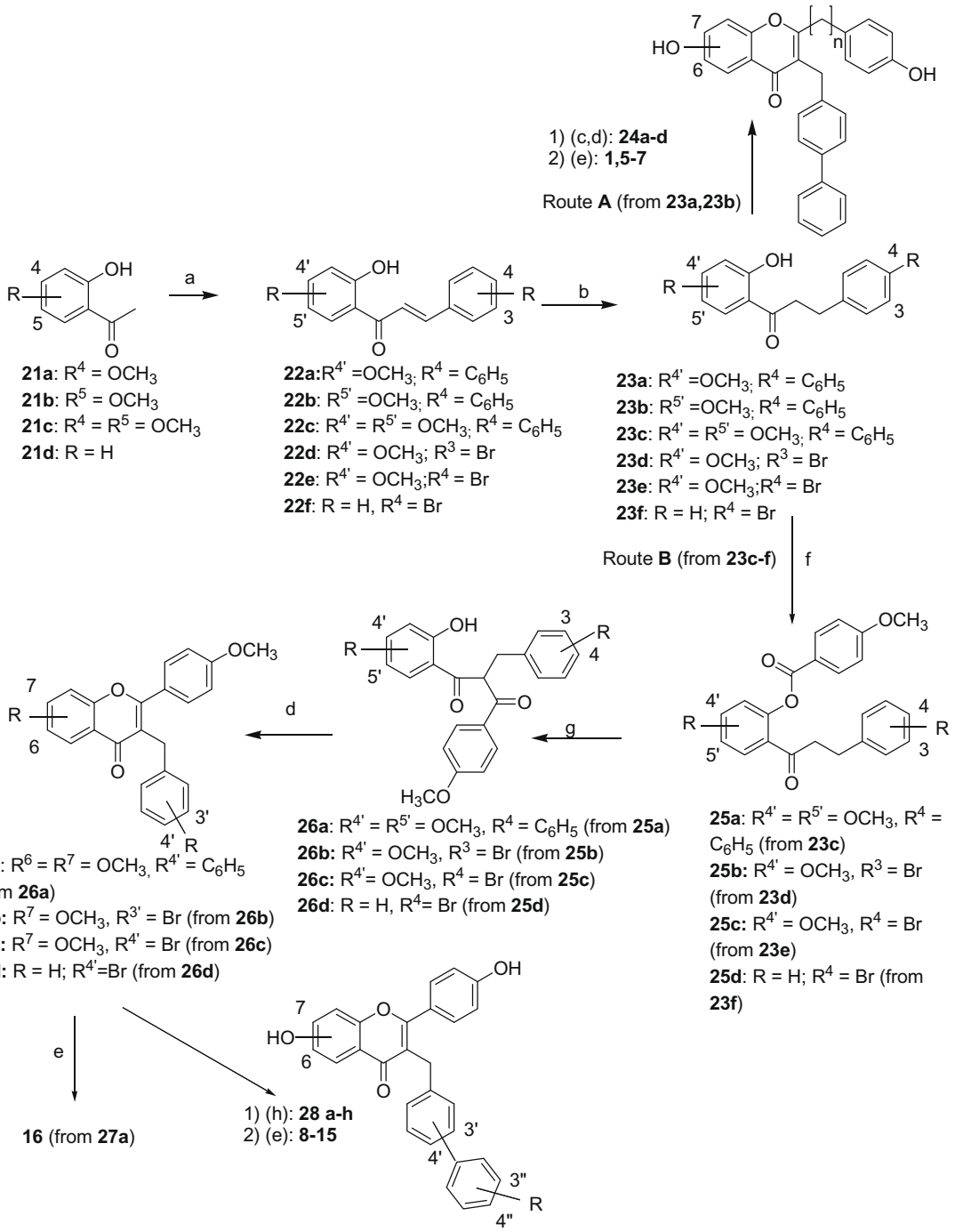
2.2. Initial screens

Screening of an in-house collection of natural and synthetic flavonoids led to the discovery of compound **1** as a lead for the development of LMW-PTP inhibitors. This compound was the only one that we found active, and it was the only one among the compounds examined that possesses a biphenyl moiety at position 3 of the flavone ring. The importance of this moiety for the enzyme inhibition was checked by the examination of compounds **2** and **3**; their lack of activity underlines the role of this moiety on the PTPases inhibitory activity of compound **1**. Moreover, compound **1** showed to be somewhat selective against PTP-1B (K_i = 2.5 μM for LMW-PTP A-form (Isoform I, IF I), K_i = 29.1 μM for PTP-1B, Table 3).

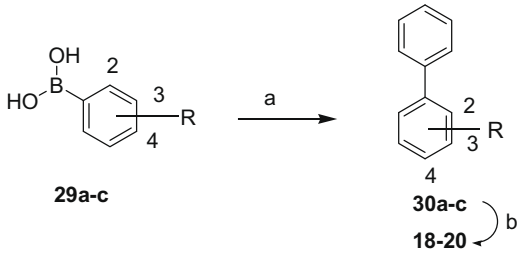
The lead was then modified at the chain at position 2 (compounds **4–6**) and at the position of the hydroxy group at position 7 (compound **7**) of the benzopyrone nucleus. The highest activity and selectivity towards PTP-1B was observed for *n* = 0 and 1, while no difference in activity was observed for the change of position of the hydroxy group from position 7 to 6 (compound **4** and **7**, respectively). Moreover, the introduction of a hydroxy group at position 4′ of the biphenyl moiety (compound **8**) influenced the inhibitory activity of compound **1** only slightly.

2.3. Molecular modeling

To gain some insight into the possible binding modes of our compounds, molecular docking simulations were performed. Since the binding site of the LMW-PTPs is lined by multiple side-chain residues that possess some conformational flexibility, we chose the Induced-Fit Docking (IFD) method by Schrödinger, LLC.[25] Initial docking experiments of compounds **1**, **4**, **7** and **8** in the active site of LMW-PTP, A-form or Isoform I (PDB code 5PNT[14]), suggested that these compounds can bind to the enzyme in two binding modes, depending on the presence of the hydroxyl group at 4′ position of the biphenyl moiety: while in the obtained poses for compounds **1**, **3** and **7**, the chromanone ring was located inside the binding pocket, for compound **8** the biphenyl was placed inside the catalytic pocket. Stacking was predicted to occur between Tyr



Scheme 1. Synthesis of compounds **1,5**–**16**: (a) (substituted)benzaldehyde, $Ba(OH)_2$, methanol, reflux; (b) Pd/C, EtOAc; (c) R-$COOCH_3$, NaH, Py; (d) CH_3COOH/H_2SO_4; (e)BBr_3; (f) 4-methoxybenzoyl chloride, TEA/DMAP; (g) NaH, DMSO; (h) Suzuki coupling, (substituted)phenylboronic acid.

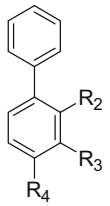


Scheme 2. Synthesis of compounds **18-20**: (a) Suzuki coupling, C_6H_5Br; (b) BBr_3.

49 and the aromatic residues that were not inside the binding pocket (details not shown).

We noticed, however, that the results obtained depended strongly on the presented protonation state of the docked compounds. Most of the PTP binding molecules have acidic groups like carboxylic acid moieties that have pK_a values well below 4.5. However, Malamas et al. reported micromolar IC_{50} values against PTP-1B for a series of benzofurane and benzothiophene biphenyls, including compounds **31–34** (Chart 3) which bear the same biphenyl residue as compound **8** and lack any other acidic group.[26] For our 7-hydroxy flavones, a pK_a of about 7.0–7.5 can be assumed[27]

Table 2
Structure and enzyme inhibition data of compounds **18–20**



Compound	R_2	R_3	R_4	IF I[a]	IF II [a]	PTP-1B[a]
18	H	OH	OH	0% inh. at 20 μM	0% inh. at 20 μM	20% inh. at 20 μM
19	H	OH	COOH	0% inh. at 20 μM	0% inh. at 20 μM	5% inh. at 20 μM
20	OH	H	OH	0% inh. at 20 μM	0% inh. at 20 μM	10% inh. at 20 μM

[a] Enzyme inhibition data are expressed as % inhibition at a given concentration.

Table 3
K_i values of selected compounds

Compound	IF I, μM (S.D.)[a]	IF II, μM (S.D.)[a]	PTP-1B, μM (S.D.)[a]
1	2.5 (0.1)[b]	7.8 (0.7)[b]	29.1 (2.2)[b]
10	2.23 (0.3)[b]	2.68 (0.1)[b]	N.D.[d]
16	0.27 (0.03)[c]	0.34 (0.06)[c]	N.D.[d]

[a] SD, Standard deviation.
[b] Mixed type inhibition.
[c] Competitive inhibition.
[d] ND, Not determined.

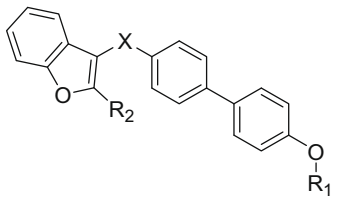
and an even higher value of 8–10, depending on possible conjugation with the flavone ring, for the phenolic hydroxy groups at the phenyl and biphenyl residues. Thus, at physiological pH, these residues can be expected to be only partially deprotonated. On the other hand, we recently performed studies on aldose reductase (ALR2) inhibitors where the assumption of deprotonated phenolic groups led to reasonable binding poses,[28] and was in good agreement with high-resolution X-ray crystal structures that suggested a hydantoin-derivative with a calculated pK_a of about 9.3 to be deprotonated at the ALR2 binding site.[29] These results showed that the protonation state of a ligand may be strongly influenced by the nature of the binding site, in addition to the pH value of the cytosol.

Available X-ray structures of PTPs showed that the residues of the phosphate binding loop are able to make multiple hydrogen bonds to oxygen atoms of phophate or sulfonate groups at the bottom of the pocket. Based on these results, we docked both 6,7-dihydroxy flavones and 3′,4′-dihydroxy biphenyl derivatives, which were both predicted to bind favorably into the binding site.

Thus, on the basis of the docking results, we decided to synthetize and test the biphenyl derivatives of general formula **A** (Chart 2) that might be able to bind to the enzyme active site via the biphenyl moiety. Based on the docking results, we prepared compounds bearing one or two hydroxy and sometimes also one carboxy group at positions 2′, 3′ and 4′, resulting in compounds **9–15** and the biphenyl derivatives **18–20** lacking the flavone residue (Tables 1 and 2, resp.). Moreover, to investigate the possible binding mode via the flavone moiety, we prepared the chromones **16** and **17** of general formula **B** (Chart 2, Table 1).

Among the compounds examined, compounds **12** and **16** showed to be the best, with an inhibitory activity (IC_{50}) towards LMW-PTP of around 1 μM. However, all these new compounds are able to inhibit LMW-PTPs and PTP-1B on approximately the same extent. Lack of activity of compounds **18–20** on all the three enzymes underlines the fact that the biphenyl, while it is very important for the activity of compound **1**, is by itself not sufficient to provide binding of the compounds. The binding affinities of **7–10** towards LMW-PTPs are virtually the same, suggesting a binding pose where substitution at the distal phenyl ring has little effect on the binding affinity, while a decreasing selectivity towards PTP-1B could be observed for every additional hydroxy or carboxy group. On the other hand, introduction of a carboxylic group at position R'_4 of compound **10** (compound **12**) gave an increase in affinity by a factor of more than 6, albeit in connection with an even higher affinity for PTP-1B. Compounds **14** and **15** showed only weak affinities.

As predicted by the docking study, the introduction of the hydroxy group at position 6 of compound **1** (compound **16**) increased the inhibitory activity by almost an order of magnitude, but, similarly to compound **12**, with lack of selectivity towards PTP-1B. Even in this case, the biphenyl moiety showed to be necessary for the activity since compound **17**, lacking this group, is practically devoid of inhibitory activity.



	X	R_1	R_2	IC_{50} (μM) PTP-1B
31	-	H	nBu	0.74
32	-	H	4-OH-Bz	1.08
33	CH_2	H	nBu	1.19
34	-	CH_2COOH	nBu	2.19

Chart 3. Inhibitory activity of some biphenyls towards PTP-1B.[26]

2.4. Docking studies

Compounds **1**, **4**, **8**, **10**, **12**, **14**, **16** and **18** were chosen for docking to give a representative sampling of our series. Additionally, quercetin and morin (Chart 1) were included.

The obtained binding profiles of the initial set of compounds suggested that binding via the 4″-hydroxyl group of the biphenyl moiety did not play a role (compounds **1** and **8** possess the same IC_{50} values, see Table 1), which appeared to be in contrast to the results obtained by Malamas et al. discussed above.[26]

Thus, given the great importance in the acidity of the inhibitors of the closely related PTP-1B for the enzyme inhibition, we calculated the pK_a values of the different hydroxy and carboxy groups, using the freely accesible web tool SPARC on line calculator v4.1[30] to gain some insights about the acidity of the different residues of the docked compounds.

This tool has been previously evaluated against experimental pK_a values of eight flavonoids—including the compounds quercetin and morin—obtained by capillary electrophoresis, and has proved to be quite successful for most predictions, with the exception that the lowest pK_a value of morin was predicted too high by 1.87 units.[31] It should be noted that all values were calculated with the intention to find out which group would be the most acidic, thus on the assumption that no other residues were already deprotonated in the molecule. Depending on the distance between the investigated residues, this simplification may have either a small or a big effect on the pK_a values of additional groups: For compound **12**, deprotonation of the most acidic carboxylic acid group does not influence the calculated value for the 7-OH position of the flavone ring (7.36) but strongly changes the value for the neighboring hydroxy group at 3″ (13.24 vs 7.31). A summary of the obtained values is given in Table 4.

The obtained values suggest that the most acidic group is—except for the carboxylic acid group in **12**—always located at position 7 of the flavone moiety. The next groups to be deprotonated would be hydroxy groups at 2-phenyl residues, preferentially at positions 2′ or 4′, followed by hydroxy groups at phenyl or biphenyl groups that are not conjugated with the flavone ring. We decided to dock the singly and—if a second pK_a value below 9.0 could be expected—doubly deprotonated form for the investigated compounds, with the exception of **18**, for which, because of its low acidity, we docked both the neutral and singly deprotonated form. A negative charge of a deprotonated 7-hydroxy flavone group can also be transferred to the oxygen atom at position 3 via mesomerism, but since these structures could not meet the main requirement of known inhibitors—a negative charge at the bottom of the narrow binding site—such mesomeric forms were not further investigated.

Table 4
Calculated and experimental pK_a values for the determination of the most acidic groups of selected docking compounds, obtained with the SPARC on line calculator[30]

Compound	Flavone ring	Residues at position 2	Residues at position 3
1	7-OH: 7.36	4′-OH: 8.61	
4	7-OH: 7.20	4′-OH: 9.84	
8	7-OH: 7.37	4′-OH: 8.62	4″-OH: 9.60
10	7-OH: 7.37	4′-OH: 8.62	4″-OH: 9.09 3″-OH: 9.30
12	7-OH: 7.36	4′-OH: 8.61	4″-COOH: 3.17 3″-OH: 7.31
14	7-OH: 7.38	4′-OH: 8.63	4″-OH: 8.99 3″-OH: 9.21
16	7-OH: 6.85 6-OH: 8.63	4′-OH: 8.65	
18	4-OH: 8.93 3-OH: 9.31		
Morin	7-OH: 7.08 5-OH: 8.42 3-OH: 10.30	2′-OH: 8.18 4′-OH: 8.48	
Exp. values[31]	5.06, 8.64, 10.62		
Quercetin	7-OH: 7.04 5-OH: 8.41 3-OH: 10.18	4′-OH: 8.26 3′-OH: 8.58	
Exp. values[31]	7.19, 9.36, 11.56		
7-Hydroxy-2-phenyl-chromen-4-one	7-OH: 7.39		
Exp. values[27]			

Calculations were performed on the assumption that the investigated group is the first one to be deprotonated (see text for discussion).

PDB structures 5PNT (human LMW-PTP isoform I[14]) and 1XWW (human LMW-PTP isoform II[32]) were selected as template structures for the docking calculations. Overlay of the two isoforms showed that the phosphate binding site is identical in both proteins. The nearest amino acid variation which may thus affect ligand selectivity is at the opening of the binding site at position 49 (Tyr/Trp for isoform I/II), followed by 50 (Glu/Asn) and 53 (Asn/Arg). The selected protein structures were processed in Maestro (Schrödinger, LLC)[25] using the Protein Preparation Wizard, which performs the following steps: assigning of bond orders, addition of hydrogens, optimization of hydrogen bonds by flipping amino side chains, correction of charges, and minimization of the protein complex. All water molecules were removed from the protein structure. The IFD protocol developed by Schrödinger, LLC, was used for docking our compounds. This method takes into account the flexibility of the binding site. The method is described in more detail elsewhere.[28,33] This approach seemed reasonable because of the flexible side chains of multiple residues reaching into the binding site, especially those of Leu 13, Ile 16, Tyr/Trp 49, Tyr 131, and Tyr 132. The size of the box, into which the compounds are docked, is usually centred around the selected co-crystallized ligand. In the case of 5PNT and 1XWW we had (a) two co-crystallized ligands (2-(*N*-morpholino)-ethanesulfonic acid (MES) and a sulfate ion) of different sizes which (b) were both considerably smaller than most of the investigated ligands. To use comparably sized binding sites that can accomodate all of our ligands, we thus chose to overlay both proteins and to copy the ligand MES from 5PNT into 1XWW. Both binding site boxes where then defined by the center of the MES ligand, with an enclosing box size set to 30 Å. All other values of the IFD protocol where left at their default values.

For each compound, the poses obtained for the two different isoforms I and II were highly similar. This is in good agreement with the rather constant IC_{50} and K_i ratio of 1.7–3.12 (Tables 1 and 3) between the affinities for isoforms I and II, suggesting that all investigated compounds share similar interactions with the amino acid residues that are different between the isoforms. One pose was predominantly found for most of our compounds (**1**, **4**, **8**, **10**, **14**, and **16**) and is depicted in Figure 2 (top row), exemplified for compound **16** in 5PNT. The flavone ring is buried inside the binding pocket, with the deprotonated 7-hydroxy group of the flavone moiety forming a charged interaction with the protonated Arg 18 residue, accepting hydrogen bonds from the same group as well as from the backbone NH group of Leu 13.

In the case of **16**, the second hydroxy group at position 6 of the flavone ring can accept additional hydrogen bonds from the surrounding backbone NH groups of Ile 16 and Cys 17, an both donate and accept a hydrogen bond from the thiol group of Cys 12. The flavone moiety is buried in the pocket lined by the side chains of Leu 13, Glu 15, Ile 16, Asp 129, and Tyr 131. While the carbonyl group at position 4 is important to raise the acidity of the 7-hydroxy group, no hydrogen bonds with the binding site residues could be observed. The 2-(4′-hydroxyphenyl) residue makes lipophilic interactions with Leu 13 and Tyr 131.

For compound **4**, this residue bends towards Tyr 49, forming hydrogen bonds between the two phenolic hydroxy groups of ligand and amino acid. Lastly, the biphenyl residue bends in such

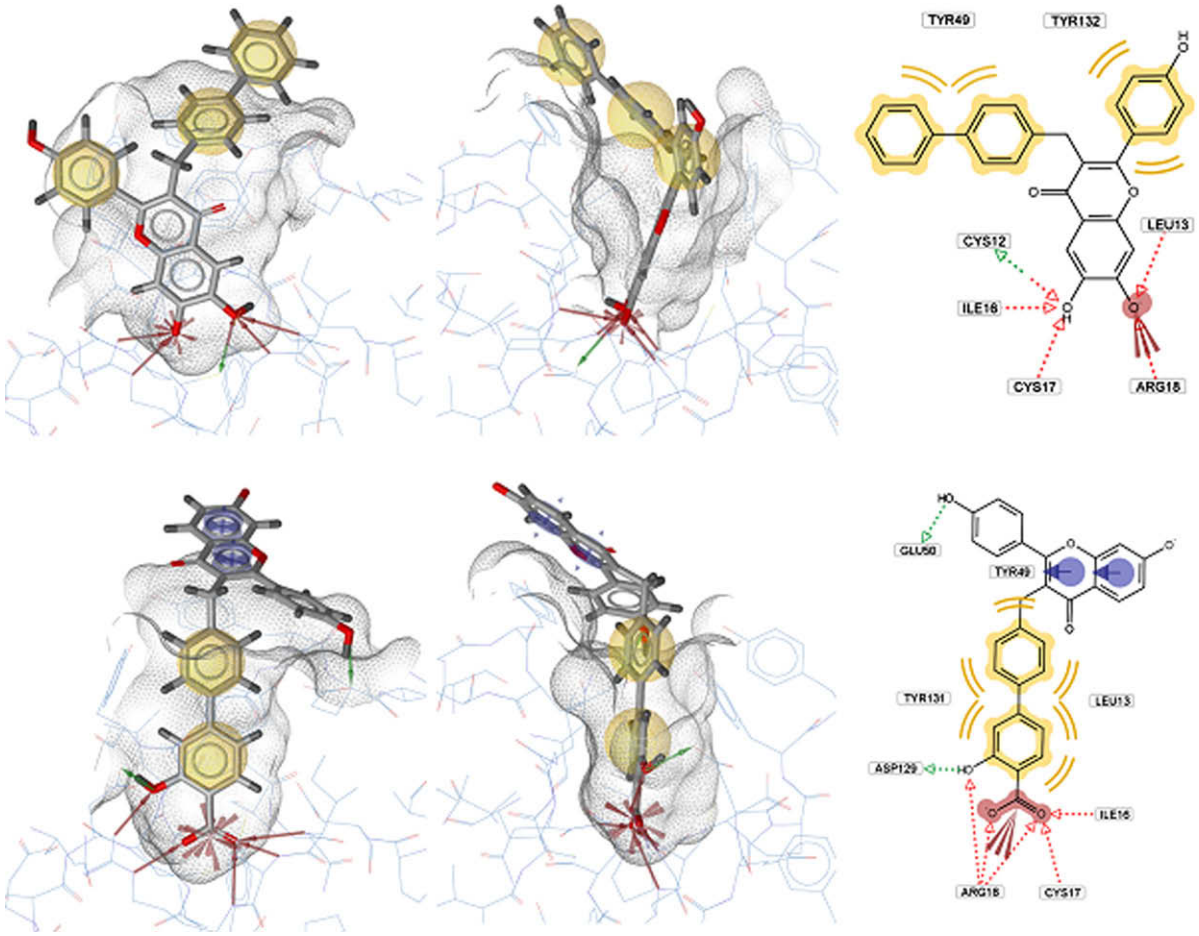


Figure 2. Docking conformations proposed for **16** (top) and **12** (bottom) in LMW-PTP isoform I (PDB structure 5PNT[14]). Left and middle: 3D representations, from two different angles, right: 2D representation. Pharmacophoric interactions were automatically detected by LIGANDSCOUT.[39] Yellow sphere: hydrophopic feature, green arrow: hydrogen bond donor, red arrow: hydrogen bond acceptor, red star: negative ionizable feature, purple ring and arrow: aromatic feature.

a way that both phenyl rings have hydrophobic interactions with the tyrosine phenyl ring. For a classical stacking, both rings are slightly shifted. Similar interactions can be observed for the indol ring of Trp 49 in the isoform II structure 1XWW (not shown). This binding pose is in good agreement with the binding data for **1**, **4**, **8**, **10**, and **16**: the compounds have similar IC_{50} values, suggesting that the structural variations occurred at positions where they had no big influence on the binding site interactions. Indeed, the presented binding pose predicts no interactions between the protein and hydroxy groups at the distal end of the biphenyl moiety, and the residues at position 2 are placed at an open part of the binding pocket, presenting possibilities for both hydrophobic and hydrogen bond interactions with residues of variable length. However, binding data for the 2-(4′-hydroxybenzyl) derivatives **4**, **5** and **6** suggest that this residue may play an important role for selectivity against PTB-1B. Likewise, additional hydroxy groups at the distal ring of the biphenyl moiety abolish selectivity against PTP-1B. For the less active compound **14**, the distal phenyl ring is shifted slightly away from the aromatic ring and towards the backbone carbonyl group of Tyr/Trp 49.

When **1**, **10**, **12** and **16** where docked in the doubly deprotonated form, bearing an additional negative charge at the 2-(4′-hydroxyphenyl) residue, also poses that placed this second negative charge at the catalystic centre were obtained. However, we were unable to correlate these poses with our binding affinity in a similarly plausible way than for the example discussed above.

Poses obtained for **12**, doubly deprotonated at both the carboxylic group and the 7-hydroxy group of the flavone, point towards a different possible binding mode for this compound (Fig. 2): Here the biphenyl moiety is buried in the catalystic pocket, with charged interactions between the carboxylic group and Arg 18, supported by hydrogen bonds donated from backbone NH groups of Ile 16, Cys 17, and Arg 18. The 3″-hydroxy group forms additional hydrogen bonds with Arg 18 and Asp 129. The biphenyl rings have hydrophobic contacts with Leu 13, Ile 16, Tyr/Trp 49, and Tyr 131, and the flavone ring stacks with the aromatic ring of Tyr/Trp 49. Lastly, the hydroxy group of the 2-(4-hydroxyphenyl) residue can donate a hydrogen bond to the carboxylate group of Glu 50 (isoform I) the guanidine group of Arg 53 (isoform II), or the backbone carbonyl group of Trp 49. Despite this alternate binding mode, a similar affinity pattern can be observed for **12** and **16**.

The biphenyl ring of **18** was placed at a similar position as that of **12**. Furthermore, the position of the 4- and 3-hydroxy groups overlaid well with that of the 7- and 6-hydroxy groups of the binding pose of **16** discussed above.

Finally, docking of morin and quercetin produced multiple different poses. In one of the two most prominent poses, the flavone ring is located in a similar way as discussed above for **16**, while in another one the flavone ring is flipped, with the carbonyl group facing the opposite direction.

Depending on the assumed protonation state, other poses where the 2-phenyl ring is buried inside the catalystic centre were

obtained as well. Because of the multiple possible tautomeric/mesomeric forms, broad distribution of hydroxy groups that are capable of forming hydrogen bonds, and the sterically undemanding character of these two molecules, no clear preference for one distinct pose could be found. While we could thus show that both morin and quercetin dock well into the investigated binding sites, we were unable to find an indication why morin is an inhibitor and quercetin is an activator of bovine LMW PTP (which, based on comparison of PDB complexes 1DG9[34] with 1XWW, has a binding site identical to that of the human isoform II).

The GlideScore values produced by the docking runs were similarly low for both the active and inactive molecules (lower negative values should indicate stronger binding interactions). For isoform I, the lowest value obtained was −9.61, while for isoform II, this value was even lower with −11.83 (morin). The problem of correlating the GlideScore values with the binding affinity can be shown by the values obtained for **18**: depending on whether the protonated or deprotonated form of the molecule was docked, the lowest GlideScore values obtained were either −9.19/−9.51 (deprotonated) or −6.75/−5.92 (protonated) for isoforms I and II, respectively. While in retrospect, this might indicate that the phenolic groups of **18** are not acidic enough to be deprotonated at the binding site, such assumptions on the protonation state—and thus on the binding affinity—are more difficult to make in prospective screening when little data is available as to which protonation states should to be expected.

Despite the considerable number of flexible protein side chains reaching into the binding pocket, most of the obtained binding site conformations showed a high similarity with the crystal structures. Compared to 5PNT, Tyr 49 was usually rotated around the bond between C_β and the phenyl ring to improve stacking with aromatic ligand residues in the calculated structures of isoform I. Additionally, Tyr 131 and Tyr 132 showed some flexibility, especially the later, where the whole side chain can rotate away from the binding site.

2.5. Cellular assays

The identification of cell-active compounds has been a major challenge in the closely related PTP-1B drug discovery programs, owing to the low cell permeability of most phosphotyrosine mimetic. Therefore the most in vitro active compounds (compounds **9**, **10**, **12**, **13** and **16**) have been assayed using a human hepatocellular liver carcinoma cell line (HepG2) which constitutively express the human insulin receptor, in order to test the compound's ability to exert an in vivo inhibition on PTPs, thus activating the insulin signalling pathway.

HepG2 cells were incubated for 90 min with **9**, **10**, **12**, **13** and **16** (10 μM final concentration) as well as with insulin (10 nM). DMSO (0.5%) and sodium orthovanadate (10 μM) were used as negative and positive controls, respectively (Fig. 3). The cell lysates were subjected to SDS-PAGE, transferred to polyvinyldene membrane, and followed by Western blot with a phospho-specific anti-IRβ antibodies (α-pIRβ) (Fig. 3A, top panel).

Among tested compounds, **10**, **12** and **16** at 10 μM concentration stimulated insulin receptor phosphorylation comparable to that exerted by insulin (Fig. 3A and B). Moreover, since Akt acts downstream to the receptor in insulin signalling and thus it is essential for the insulin-stimulated Glut4 translocation to the plasma membrane, agents that enhance activity of Akt should enhance cellular glucose uptake. Thus blots were also re-probed with a phospho-specific anti-Akt antibody (α-pAkt). Figure 3A (middle panel) shows that compounds **10** and **12** at 10 μM concentration are capable to increase the phosphorylation level of Akt with an extent very close to that exerted by 10 nM insulin. In addition, the same blots were re-probed with an anti-phosphotyrosine antibody. Figure 3C demonstrates that insulin and the above compounds increase tyrosine phosphorylation of identical protein bands, whereas 10 μM orthovanadate, a general PTP inhibitor, causes promiscuous tyrosine phosphorylation (Fig. 3D).

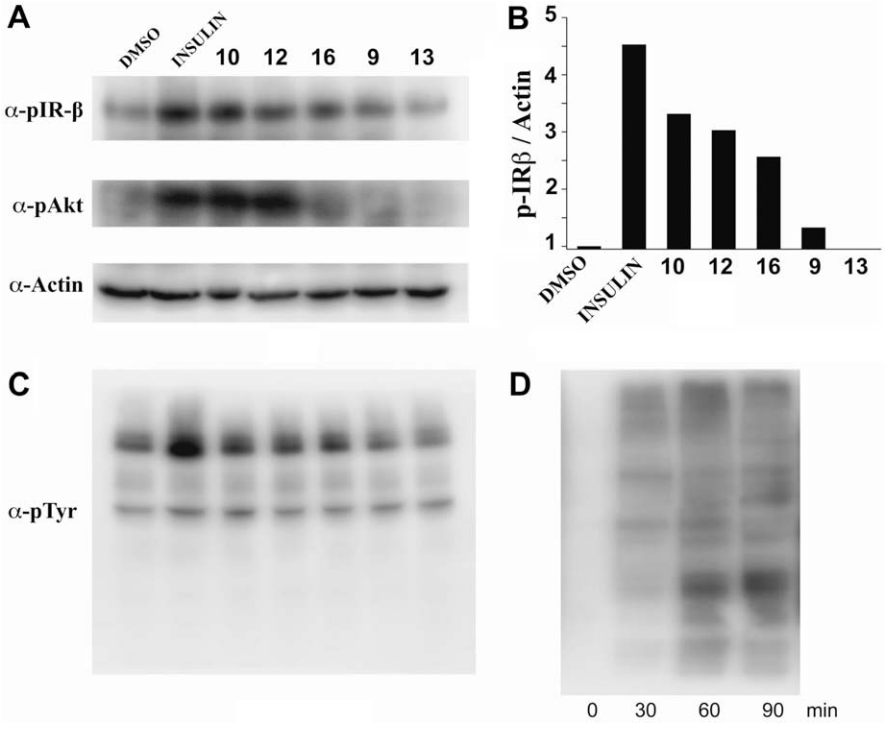


Figure 3. Effect of **9**, **10**, **12**, **13** and **16** and insulin on tyrosine phosphorylation of IR in HepG2 cells. Subconfluent HepG2 cells were incubated at 37 °C with these compounds at 10 μM final concentration as well as with 10 nM insulin for 90 min. The cell lysates were subjected to SDS-PAGE and the resolved polypeptides transferred to polyvinylidene membranes, which were probed with anti-phospho-insulin receptor β-subunit (α-pIRβ). Then membrane was stripped and reprobed with anti-phospho-Akt (α-Akt), anti-phosphotyrosine (α-pTyr), and anti-actin (α-Actin) antibodies. Data are representative of at least three separate experiments.

3. Conclusions

The docking poses presented in the previous section appear well fit to explain the SAR of our compounds on a qualitative level. However, the difference in acidity of the phenolic hydroxyls is too little in order to decide unequivocally which is the binding mode of the compounds inside the active site; the two most probably binding poses (Fig. 2) will be useful for the design of new compounds in order to investigate the accurateness of the presented models. Moreover, while we were yet unable to obtain substantial selectivity for one of the two human isoforms of LMW-PTP, the docking results provide a good starting point to further derivative our compounds to let them interact with the subtype diverse side chain residues at amino acid positions 49, 50, and 53. Some of the most active compounds in in vitro assays were shown to be active on cells, being able to increase the extent of IRβ tyrosine phosphorylation in HepG2 cells and activate Akt (Fig. 3); the lack of activity of **13,** which is active in vitro, is at the moment unexplained.

In conclusion, a new class of highly active LMW-PTP inhibitors was discovered. Docking experiments and experimental results highlighted the possible binding mode of these compounds to the enzyme, providing the background for the future optimization of their inhibitory activity and selectivity towards the closely related enzyme PTP-1B.

4. Experimental

4.1. Chemistry

Melting points were determined on a Buchi 510 melting point apparatus and are uncorrected. NMR spectra were recorded on a Bruker DPX200 or Avance400 spectrometers (Centro Interdipartimentale Grandi Scrumenti, Modena University). Chemical shifts are reported in ppm from tetramethylsilane. *J* values are given in Hertz. Microanalyses were carried out in the microanalysis laboratory of the Dipartimento di Scienze Farmaceutiche, Modena University. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. TLC were performed on precoated silica gel F254 plates (Merck). Silica gel (Merck, 70–230 mesh) was used for column chromatography. Mass spectra were obtained on a Finnigan MAT SSQ 7000 spectrometer (EI, 70 eV). Compounds **2–4** and **17** were available from previous researches.[24] Chalchones **22a**[24], **22f**[35] and **23a**[24] were synthesized as described.

4.1.1. General synthesis of compounds 22a–e

To a solution of the appropriate acetophenone **21a–d** (18.0 mmol) and the appropriate (substituted) benzaldehyde (18.0 mmol) in CH_3OH (100 mL) a solution of $Ba(OH)_2$ (3.42 g, 18.0 mmol) was added dropwise during 15 min under stirring at rt; the solution thus obtained was heated to reflux for 2 h; after cooling, the solvent was removed under reduced pressure and the residue was diluted with water (100 mL), acidified (pH 5.0) with 1 N HCl, and extracted with EtOAc (3 × 50 mL). The organic phase was then dried (Na_2SO_4), the solvent removed under reduced pressure and the residue crystallized from EtOH.

4.1.1.1. 2′-Hydroxy-4-phenyl-4′-methoxychalchone (22a). Yield 90%, mp 103–5 °C (EtOH); ^{1}H NMR ($CDCl_3$): 13.50 (1H, broad s), 7.97 (1H, d, *J* 15.5), 7.89 (2H, m), 7.80–7.40 (9H, m), 6.55 (1H, dd, *J* 8.2, *J* 2.1), 6.53 (1H, s), 3.96 (3H, s). Anal. ($C_{22}H_{18}O_3$) C, H.

4.1.1.2. 2′-Hydroxy-4-phenyl-5′-methoxychalchone (22b). Compound **22b** was synthesized by reaction between 2′-hydroxy-5′-methoxyacetophenone and 4-biphenylcarboxaldehyde: yield 66%; mp 118–20 °C; ^{1}H NMR ($CDCl_3$): 12.40 (1H, broad s), 7.97 (1H, d, *J* 15.5), 7.80–7.59 (7H, m), 7.56–7.35 (4H, m), 7.16 (1H, dd, *J* 3.9, *J* 9.1), 7.00 (1H, d, *J* 9.1), 3.89 (3H, s). Anal. ($C_{22}H_{18}O_3$) C, H.

4.1.1.3. 2′-Hydroxy-4-phenyl-4′,5′-dimethoxychalchone (22c). Compound **22c** was synthesized by reaction between 2′-hydroxy-4′,5′-dimethoxyacetophenone and 4-biphenylcarboxaldehyde: yield 71%; mp 140–2 °C; ^{1}H NMR ($CDCl_3$): 13.38 (1H, broad s), 7.94 (1H, d, *J* 15.4), 7.84–7.23 (11H, m), 6.55 (1H, s), 3.96 (3H, s), 3.94 (3H, s). Anal. ($C_{23}H_{20}O_4$) C, H.

4.1.1.4. 3-Bromo-2′-hydroxy-4′-methoxychalchone (22d). Compound **22d** was synthesized by reaction between 2′-hydroxy-4′-methoxyacetophenone and 3-bromobenzaldehyde: yield 77%, mp 98–100 °C; ^{1}H NMR ($CDCl_3$): 13.31 (1H, broad s), 7.85 (3H, m), 7.60 (3H, m), 7.32 (1H, d, *J* 7.1), 6.51 (1H, dd, *J* 2.5, *J* 8.7), 6.49 (1H, s), 3.88 (3H, s). Anal. ($C_{16}H_{13}BrO_3$) C, H.

4.1.1.5. 4-Bromo-2′-hydroxy-4′-methoxychalchone (22e). Compound **22e** was synthesized by reaction between 2′-hydroxy-4′-methoxyacetophenone and 4-bromobenzaldehyde: yield 56%, mp 138–40 °C; ^{1}H NMR ($CDCl_3$): 13.35 (1H, broad s), 7.89–7.78 (2H, m), 7.64–7.40 (5H, m), 6.50 (1H, dd, *J* 2.5, *J* 7.3), 6.48 (1H, s), 3.88 (3H, s). Anal. ($C_{16}H_{13}BrO_3$) C, H.

4.1.2. General synthesis of compounds 23a–f

A solution of the appropriate chalchone **22a–f** (3.0 mmol) and Pd/C (0.6 g) in EtOAc (70 mL) was hydrogenated at 4 atm. for 1 h. The reaction mixture was filtered through Celite and the filtrate was evaporated under reduced pressure to give a solid, which was crystallized (MeOH, unless otherwise stated).

4.1.2.1. 2′-Hydroxy-4-phenyl-4′-methoxydihydrochalchone (23a). Yield 92%, mp 100–2 °C (EtOH) (103–4 °C)[36]; ^{1}H NMR ($CDCl_3$): 7.72–7.29 (10H, m), 6.47 (1H, s), 6.44 (1H, dd, *J* 2.5, *J* 7.3), 3.73 (3H, s), 3.30 (2H, m), 3.15 (2H, m). Anal. ($C_{22}H_{20}O_3$) C, H.

4.1.2.2. 4-Phenyl-2′-hydroxy-5′-methoxydihydrochalchone (23b). Yield 88%; mp 76–8 °C; ^{1}H NMR ($CDCl_3$): 7.63–7.30 (9 H, m), 7.20 (1H, d, *J* 3.2), 7.14 (1H, dd, *J* 3.2, *J* 8.9), 6.96 (1H, d, *J* 8.9), 3.80 (3H, s), 3.38 (2H, m), 3.15 (2H, m). Anal. ($C_{22}H_{20}O_3$) C, H.

4.1.2.3. 4-Phenyl-2′-hydroxy-4′,5′-dimethoxydihydrochalchone (23c). Yield 79%; mp 83–5 °C; ^{1}H NMR ($CDCl_3$): 12.72 (1H, broad s), 7.65–7.30 (9H, m), 7.08 (1H, s), 6.49 (1H, s), 3.93 (3H, s), 3.82 (3H, s), 3.30 (2H, m), 3.15 (2H, m). Anal. ($C_{23}H_{22}O_4$) C, H.

4.1.2.4. 3-Bromo-2′-hydroxy-4′-methoxydihydrochalchone (23d). Yield 81%; mp 61–3 °C; ^{1}H NMR ($CDCl_3$): 12.71 (1H, broad s), 7.63 (1H, d, *J* 9.6), 7.45–7.10 (4H, m), 6.49–6.38 (2H, m), 3.85 (3H, s), 3.23 (2H, m), 3.05 (2H, m). Anal. ($C_{16}H_{15}BrO_3$) C, H.

4.1.2.5. 4-Bromo-2′-hydroxy-4′-methoxydihydrochalchone (23e). Yield 89%, mp 44–6 °C; ^{1}H NMR ($CDCl_3$): 12.73 (1H, broad s), 7.62 (2H, d, *J* 9.5), 7.42 (2H, d, *J* 8.3), 7.12 (2H, d, *J* 8.3), 6.45 (2H, m), 3.85 (3H, s), 3.21 (2H, m), 3.01 (2H, m). Anal. ($C_{16}H_{15}BrO_3$) C, H.

4.1.2.6. 4-Bromo-2′-hydroxydihydrochalchone (23f). Yield 86%; mp70–2 °C; ^{1}H NMR ($CDCl_3$): 7.70 (1H, dd, *J* 2.1, *J* 8.0); 7.56–7.38 (3H, m), 7.16 (2H, m), 7.00 (1H, dd, *J* 1.4, *J* 8.0), 6.90 (1H, m), 3.32 (2H, m), 3.05 (2H, m). Anal. ($C_{15}H_{13}BrO_2$) C, H.

4.1.3. General synthesis of compounds 24a–d

A solution of the appropriate dihydrochalchone **23a–b** (3.01 mmol) and the appropriate methyl ester (3.31 mmol, 1.1 equiv) in anhyd pyridine (9 mL) was added dropwise to a

well-stirred suspension of NaH (60% dispersion in mineral oil) (9.03 mmol, 3.0 equiv) in anh. pyridine (7 mL). When the reaction subsided, the mixture was heated at 90 °C for 15 min. After cooling, the mixture was decomposed in 2 N HCl and extracted with methylene chloride (2 × 60 mL). The combined organic layers were washed with 1 N HCl (2 × 30 mL) and water (30 mL), then dried (Na_2SO_4), and the solvent was removed under reduced pressure. The residue was purified by means of column chromatography (cyclohexane/ethyl acetate 8.5/1.5); the oily product thus obtained showed an R_f 0.15–0.20 (cyclohexane/ethyl acetate 8.5/1.5). The product was then dissolved in acetic acid (20 mL) and concentrated sulphuric acid (0.1 mL). The resulting solution was then heated at 100 °C for 1 h. After cooling, the solution was neutralized with $NaHCO_3$ 2 N then extracted with ethyl acetate (2 × 30 mL), the organic layer was washed again with $NaHCO_3$ 0.5 N and satd NaCl solution, dried (Na_2SO_4) and the solvent was removed under reduced pressure. The residue was then washed with cold water and collected by filtration.

4.1.3.1. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (24a). Compound **24a** was synthesized by reaction between **23a** and 4-methoxyphenylbenzoic acid methyl ester. Yield 12%; ^{1}H NMR ($CDCl_3$): 8.18 (1H, d, *J* 8.9), 7.60–7.22 (11H, m), 7.00 (2H, d, *J* 8.9), 7.00 (1H, dd, *J* 2.4, *J* 8.9), 6.87 (1H, d, *J* 2.4), 4.03 (2H, s), 3.91 (3H, s), 3.87 (3H, s). Anal. ($C_{30}H_{24}O_4$) C, H.

4.1.3.2. 7-Methoxy-2-(4′-methoxyphenylethyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (24b). Compound **24b** was synthesized by reaction between **23a** and 4-methoxyphenylpropanoic acid methyl ester. Yield 18%; ^{1}H NMR ($CDCl_3$): 8.16 (1H, d, *J* 8.9), 7.60–7.21 (9H, m), 7.04 (2H, d, *J* 8.4), 6.97 (1H, dd, *J* 1.9, *J* 8.9), 6.83 (1H, s), 6.81 (2H, d, *J* 8.4), 3.92 (3H, s), 3.88 (2H, s), 3.78 (3H, s), 2.94 (4H, m). Anal. ($C_{32}H_{28}O_4$) C, H.

4.1.3.3. 7-Methoxy-2-(4′-methoxyphenylproyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (24c). Compound **24c** was synthesized by reaction between **23a** and 4-methoxyphenylbutanoic acid methyl ester. Yield 22%; ^{1}H NMR ($CDCl_3$): 8.15 (1H, d, *J* 8.9), 7.60–7.18 (9H, m), 7.05 (2H, d, *J* 8.4), 6.96 (1H, dd, *J* 1.8, *J* 8.9), 6.81 (2H, d, *J* 8.4), 6.80 (1H, s), 3.90 (6H, s), 3.78 (2H, s), 2.72 (2H, t, *J* 7.7), 2.62 (2H, t, *J* 7.5), 1.95 (2H, m). Anal. ($C_{33}H_{30}O_4$) C, H.

4.1.3.4. 6-Methoxy-2-(4′-methoxybenzyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (24d). Compound **24d** was synthesized by reaction between **23b** and 4-methoxyphenylacetic acid methyl ester. Yield 25%; ^{1}H NMR ($CDCl_3$): 7.65–7.18 (12H, m), 7.10 (2H, d, *J* 8.7), 6.82 (2H, d, *J* 8.7), 4.09 (2H, s), 4.03 (2H, s), 3.90 (3H, s), 3.78 (3H, s). Anal. ($C_{31}H_{26}O_4$) C, H.

4.1.4. General synthesis of the phenylesters (25a–d)

To a stirred solution of the appropriate dihydrochalchone **23c–f** (3.0 mmol), triethylamine (6.0 mmol, 2 equiv) and dimethylaminopyridine (0.3 mmol, 0.1 equiv) in 5 mL of methylene chloride at 0 °C was added dropwise 4-methoxybenzoyl chloride (2 mL, 3.3 mmol, 1.1 equiv). The reaction mixture was allowed to warm to rt over 1 h, then the solution was diluted with methylene chloride and washed successively with 1 N HCl and water, then dried (Na_2SO_4) and concentrated under reduced pressure. The residue thus obtained was triturated with diethyl ether (5 mL), and the residue was collected by filtration.

4.1.4.1. 4′,5′-Dimethoxyphenyl-2-[3-(biphenyl)propionyl]-4-methoxyphenyl ester (25a) (from 23c). Yield 85%; ^{1}H NMR ($CDCl_3$): 8.13 (2H, d, *J* 8.9), 7.60–7.29 (8H, m), 7.12 (2H, d, *J* 8.1), 6.94 (2H, d, *J* 8.9), 6.71 (1H, s), 3.94 (6H, s), 3.83 (3H, s), 3.23 (2H, m), 3.01 (2H, m). Anal. ($C_{31}H_{28}O_6$) C, H.

4.1.4.2. 4′-Methoxyphenyl-2-[3-(3-bromophenyl)propionyl]-4-methoxy phenyl ester (25b) (from 23d). Yield 79%; mp 40–3 °C, ^{1}H NMR ($CDCl_3$): 8.15 (2H, m), 7.87 (1H, d, *J* 8.8), 7.30–6.92 (6H, m), 6.86 (1H, dd, *J* 2.5, *J* 8.8), 6.73 (1H, d, *J* 2.5), 3.92 (3H, s), 3.86 (3H, s), 3.15 (2H, m), 2.94 (2H, m). Anal. ($C_{24}H_{21}BrO_5$) C, H.

4.1.4.3. 4′-Methoxyphenyl-2-[3-(4-bromophenyl)propionyl]-4-methoxy phenyl ester (25c) (from 23e). Yield 88%; mp 65–7 °C, ^{1}H NMR ($CDCl_3$): 8.12 (2H, m), 7.85 (1H, d, *J* 8.7), 7.30 (2H, m), 7.03–6.90 (4H, m), 6.87 (1H, dd, *J* 2.3, *J* 8.7), 6.73 (1H, d, *J* 2.3), 3.92 (3H, s), 3.88 (3H, s), 3.88 (3H, s), 3.15 (2H, m), 2.92 (2H, m). Anal. ($C_{24}H_{21}BrO_5$) C, H.

4.1.4.4. Phenyl-2-[3-(4-bromophenyl)propionyl]-4-methoxy phenyl ester (25d) (from 23f). Yield 77%; mp 108–10 °C; ^{1}H NMR ($CDCl_3$):8.10 (2H, m), 7.76 (1H, dd, *J* 2.3, *J* 8.1), 7.57 (1H, m), 7.40–7.20 (4H, m), 7.06–6.90 (4H, m), 3.91 (3H, s), 3.20 (2H, m), 2.94 (2H, m). Anal. ($C_{23}H_{19}BrO_4$) C, H.

4.1.5. General synthesis of 4*H*-1-benzopyran-4-one derivatives (27a–d)

To a stirred suspension of NaH (60%, 6 mmol, 3 equiv) in DMSO (2 mL) at 20 °C was added dropwise a solution of **25a–d** (2.0 mmol) in DMSO (3.5 mL). The reaction mixture was stirred at 20 °C for 3 h, then slowly poured into a stirred slurry of ice in an oxalic acid solution. The aqueous mixture was extracted with ethyl acetate which in turn was successively washed with water and dried (Na_2SO_4) and concentrated under reduced pressure to give an oil that was purified by column chromatography (cyclohexane/ethyl acetate 7.5/2.5) (**26a–d**). The ^{1}H NMR spectra ($CDCl_3$) showed the characteristic presence of a triplet (1H, t, *J* = 6.9) at 5.30 ppm. The oil was then dissolved in CH_3COOH (10 mL) and H_2SO_4 (0.1 mL), then it was heated at 95 °C for 1 h. After cooling, the solution was neutralized with $NaHCO_3$ 2 N and then extracted with ethyl acetate (2 × 20 mL). The organic layer was rewashed with $NaHCO_3$ 0.5 N and saturated NaCl solution, then dried (Na_2SO_4) and the solvent was removed under reduced pressure.

4.1.5.1. 2-(4-Methoxyphenyl)-3-(biphenylmethyl)-6,7-dimethoxy-4*H*-1-benzopyran-4-one (27a) (from 26a). Yield 55%; mp 200–2 °C; ^{1}H NMR (DMSO-d_6): 7.70–7.30 (10H, m), 7.24 (1H, s), 7.16 (2H, d, *J* 8.4), 7.08 (2H, m), 3.92 (3H, s), 3.87 (3H, s), 3.82 (3H, s), 3.30 (2H, s). Anal. ($C_{31}H_{26}O_5$) C, H.

4.1.5.2. 2-(4-Methoxyphenyl)-3-(3-bromobenzyl)-7-methoxy-4*H*-1-benzopyran-4-one (27b) (from 26b). Yield 49%; mp 60–3 °C; ^{1}H NMR ($CDCl_3$): 8.16 (1H, d, *J* 8.4), 7.49 (2H, m), 7.25 (2H, m), 7.10 (2H, m), 7.05–6.95 (3H, m), 6.88 (1H, d, *J* 2.1), 3.94 (2H, s), 3.91 (3H, s), 3.88 (3H, s). Anal. ($C_{24}H_{19}BrO_4$) C, H.

4.1.5.3. 2-(4-Methoxyphenyl)-3-(4-bromobenzyl)-7-methoxy-4H-1-benzopyran-4-one (27c) (from 26c). Yield 45%; mp 145–7 °C; ^{1}H NMR ($CDCl_3$): 8.15 (1H, d, *J* 8.9), 7.48 (2H, d, *J* 8.9), 7.35 (2H, d, *J* 8.4), 7.10–6.93 (5H, m), 6.87 (1H, d, *J* 2.3), 3.92 (5H, s), 3.88 (3H, s). Anal. ($C_{24}H_{19}BrO_4$) C, H.

4.1.5.4. 2-(4-Methoxyphenyl)-3-(4-bromobenzyl)-4*H*-1-benzopyran-4-one (27d) (from 26d). Yield 49%; mp 108–10 °C; ^{1}H NMR ($CDCl_3$): 8.25 (1H, dd, *J* 2.1, *J* 8.6), 7.68 (1H, m), 7.55–7.30 (6H, m), 7.10–6.95 (4H, m), 3.95 (2H, s), 3.81 (3H, s). Anal. ($C_{23}H_{17}BrO_3$) C, H.

4.1.6. General synthesis of compounds 28a–h

To a suspension of $Pd(PPh_3)_4$ (0.039 mmol, 0.05 equiv) in anhydrous DME (2 mL) was added the compound **27b** (for compounds **14** and **15**), **27c** (for compounds **9**, **10** and **12**) and **27d** (for compounds **11** and **13**) (0.35 g, 0.78 mmol) in DME (5.5 mL) and the solution thus obtained was stirred for 20 min at rt. Then the appropriate phenylboronic acid (1.17 mmol, 1.5 equiv) in DME (2.5 mL) was added and the mixture became dark and cloudy. After adding K_2CO_3 (1.56 mmol, 2 equiv) as solid, the reaction was refluxed overnight. After cooling, the suspension was diluted with water (60 mL) and saturated NaCl solution (30 mL), then extracted with EtOAc (2 × 60 mL). The combined organic layers were washed with saturated NaCl solution and dried over $MgSO_4$, then concentrated under reduced pressure. The residue was purified by column chromatography (*n*-hexane/EtOAc 6/4).

4.1.6.1. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(4″-methoxy-1,1′-biphenyl-4-yl)methyl]-4H-1-benzopyran-4-one (28a). Compound **28a** was synthesized by reaction between **27c** and 4-methoxyphenyl boronic acid: yield 30%; ^{1}H NMR ($CDCl_3$): 8.18 (1H, d, *J* 8.9), 7.60–7.40 (6H. m), 7.21 (2H, d, *J* 8.3), 7.05–6.92 (5H, m), 6.87 (1H, d, *J* 2.3), 4.02 (2H, s), 3.90 (3H, s), 3.86 (3H, s), 3.83 (3H, s). Anal. ($C_{31}H_{26}O_5$) C, H.

4.1.6.2. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(3″-carboxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one ethyl ester (28b). Compound **28b** was synthesized by reaction between **27c** and 3-carboxyphenyl boronic acid ethyl ester: yield 46%; ^{1}H NMR ($CDCl_3$): 8.25–7.35 (8H, m), 7.30–6.85 (7H, m), 4.41 (2H, q, *J* 7.1), 4.03 (2H, s), 3.91 (3H, s), 3.88 (3H, s), 1.42 (3H, t, *J* 7.1). Anal. ($C_{33}H_{28}O_6$) C, H.

4.1.6.3. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(3″,4″-dimethoxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (28c). Compound **28c** was synthesized by reaction between **27c** and 3,4-dimethoxyphenyl boronic acid: yield 68%; mp 153–5 °C; ^{1}H NMR ($CDCl_3$): 8.18 (1H, d, *J* 8.9), 7.56 (2H, d, *J* 8.9), 7.43 (2H, d, *J* 8.2), 7.22 (2H, d, *J* 8.2), 7.18–6.85 (7H, m), 4.01 (2H, s), 3.94 (3H, s), 3.91 (6H, s), 3.88 (3H, s). Anal. ($C_{32}H_{28}O_6$) C, H.

4.1.6.4. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(4″carboxy-3″-methoxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one methyl ester (28d). Compound **28d** was synthesized by reaction between **27c** and 4-carboxy-3-hydroxyphenyl boronic acid methyl ester: yield 36%; mp 140–2 °C; ^{1}H NMR ($CDCl_3$): 8.16 (1H, d, *J* 8.9), 7.86 (1H, d, *J* 7.9), 7.55 (2H, d, *J* 8.8), 7.49 (2H, d, *J* 8.2), 7.30–6.95 (7H, m), 6.88 (1H, d, *J* 2.4), 4.03 (2H, s), 3.96 (3H, s), 3.92 (3H, s), 3.91 (3H, s), 3.88 (3H, s). Anal. ($C_{33}H_{28}O_7$) C, H.

4.1.6.5. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(3″,4″-dimethoxy-1,1′-biphenyl-3-yl)methyl]-4*H*-1-benzopyran-4-one (28e). Compound **28e** was synthesized by reaction between **27b** and 3,4-dimethoxyphenyl boronic acid: yield 71%; mp 105–7 °C; ^{1}H NMR ($CDCl_3$): 8.16 (1H, d, *J* 8.8), 7.53 (2H, d, *J* 7.6), 7.40–6.80 (11H, m), 4.05 (2H, s), 3.93 (3H, s), 3.92 (6H, s), 3.85 (3H, s). Anal. ($C_{32}H_{28}O_6$) C, H.

4.1.6.6. 7-Methoxy-2-(4′-methoxyphenyl)-3-[(4″-carboxy-3″-methoxy-1,1′-biphenyl-3-yl)methyl]-4*H*-1-benzopyran-4-one methyl ester (28f). Compound **28f** was synthesized by reaction between **27b** and 4-carboxy-3-methoxyphenyl boronic acid methyl ester: yield 21%; mp 53–5 °C; ^{1}H NMR ($CDCl_3$): 8.16 (1H, d, *J* 8.8), 7.83 (1H, d, *J* 7.8), 7.53 (2H, d, *J* 8.4), 7.40–6.90 (9H, m), 6.86 (1H, d, *J* 2.0), 4.06 (2H, s), 3.93 (3H, s), 3.90 (6H, s), 3.85 (3H, s). Anal. ($C_{33}H_{28}O_7$) C, H.

4.1.6.7. 2-(4′-Methoxyphenyl)-3-[(3″,4″-dimethoxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (28g). Compound **28g** was synthesized by reaction between **27d** and 3,4-dimethoxyphenyl boronic acid: yield 77%; mp 45–47 °C; ^{1}H NMR ($CDCl_3$): 8.27 (1H, dd, *J* 1.9, *J* 8.1), 7.68 (1H, m), 7.58 (2H, m), 7.53–7.35 (4H, m), 7.22 (2H, m), 7.16–6.90 (5H, m), 4.06 (2H, s), 3.93 (3H, s), 3.91 (3H, s), 3.87 (3H, s). Anal. ($C_{31}H_{26}O_5$) C, H.

4.1.6.8. 2-(4′-Methoxyphenyl)-3-[(4″carboxy-3″-methoxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one methyl ester (28h). Compound **28h** was synthesized by reaction between **27d** and 4-carboxy-3-methoxyphenyl boronic acid methyl ester: yield 42%; mp 48–50 °C; ^{1}H NMR ($CDCl_3$): 8.28 (1H, dd, *J* 2.2, *J* 8.0), 7.86 (1H, d, *J* 8.0), 7.69 (1H, m), 7.58 (2H, m), 7.50 (2H, m), 7.42 (1H, m), 7.32–7.12 (5H, m), 7.01 (2H, m), 4.06 (2H, s), 3.96 (3H, s), 3.91 (3H, s), 3.89 (3H, s). Anal. ($C_{32}H_{26}O_6$) C, H.

4.1.7. Synthesis of the biphenyl derivatives 30a–c

A mixture of Na_2CO_3 (212 mg, 2 mmol), the appropriate phenylboronic acid **29a–c** (1.5 mmol), $Pd(OAc)_2$ (1.1 mg, 0.005 mmol) and bromobenzene (105 μL, 1 mmol) in distilled water/acetone (3.5 and 3 mL, respectively) was stirred for 1 h at 35 °C in a water bath. The reaction was then extracted with EtOAc (2 × 10 mL). The combined organic layers were washed with saturated NaCl solution and dried over $MgSO_4$, then concentrated under reduced pressure. The residue was purified by column chromatography (n-hexane/EtOAc 6/4).

4.1.7.1. 3′,4′-Dimethoxy-1,1′-biphenyl (30a). Compound **30a** was synthesized by reaction between bromobenzene and 3,4-dimethoxyphenyl boronic acid: yield 75%; mp 58–60 °C; ^{1}H NMR ($CDCl_3$): 7.62–7.53 (2H, m), 7.50–7.28 (3H, m), 7.22–7.10 (2H, m), 6.96 (1H, d, *J* 8.1), 3.97 (3H, s), 3.94 (3H, s). Anal. ($C_{14}H_{14}O_2$) C, H.

4.1.7.2. 4′-Carboxy-3′-methoxy-1,1′-biphenyl methyl ester (30b). Compound **30b** was synthesized by reaction between bromobenzene and 4-carboxy-3-methoxyphenyl boronic acid methyl ester: yield 74%; mp 45–7 °C; ^{1}H NMR ($CDCl_3$): 7.90 (1H, d, *J* 7.9), 7.68–7.58 (2H, m), 7.54–7.40 (3H, m), 7.25–7.16 (2H, m), 4.01 (3H, s), 3.93 (3H, s). Anal ($C_{15}H_{14}O_3$) C, H.

4.1.7.3. 2′,4′-Dimethoxy-1,1′-biphenyl (30c). Compound **30c** was synthesized by reaction between bromobenzene and 2,4-dimethoxyphenyl boronic acid: yield 93% (oil); ^{1}H NMR ($CDCl_3$): 7.60–7.26 (6H, m), 6.58 (2H, m), 3.88 (3H, s), 3.83 (3H, s). Anal. ($C_{14}H_{14}O_2$) C, H.

4.1.8. Synthesis of compounds 1, 5–16 and biphenyl derivatives 18–20

A solution of the protected compounds **24a–d**, **27a**, **28a–h** and **30a–c** (0.5 mmol) in methylene chloride (10 mL) at 0 °C was treated with BBr_3 (1.0 M solution in CH_2Cl_2) (2 equiv for each methoxy-group). The resulting solution was stirred at rt for 24 h, then cooled at 0 °C, water and ice were added, and the precipitate thus formed was filtrated and washed with water. Column chromatography (CH_2Cl_2/CH_3OH 9:1 for compounds **1**, **5–8**, **10**, **11**, **14** and **16**, CH_2Cl_2/CH_3OH 9:1 followed by CH_3OH 100% for compounds **9**,**12**,**13** and **15**, cyclohexane/ethyl acetate 8:2 for **18** and **20**) allowed the obtainment of the desired compounds.

4.1.8.1. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (1) (from 24a). Yield 68%; mp 247–50 °C dec (Methanol); ^{1}H NMR (DMSO-d_6): 10.71 (1H, broad s), 10.0 (1H, broad s), 7.90 (1H, d, *J* 8.6), 7.67–7.30 (9H, m), 7.15 (2H, d, *J* 8.2), 6.90 (4H, m), 3.88 (2H, s). Anal. ($C_{28}H_{20}O_4$) C, H.

4.1.8.2. 7-Hydroxy-2-(4′-hydroxyphenyletyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (5) (from 24b). Yield 71%; mp 195–7 °C (dec.); ^{1}H NMR (400 MHz, DMSO-d_6): 7.86 (1H, d, *J* 8.7), 7.59 (2H, d, *J* 7.7), 7.50 (2H, d, *J* 8.1), 7.42 (2H, dd, *J* 7.4, *J* 7.7), 7.32 (1H, t, *J* 7.4), 7.19 (2H, d, *J* 8.1), 6.94 (2H, d, *J* 8.4), 6.90 (1H, dd, *J* 2.1, *J* 8.7), 6.84 (1H, d, *J* 2.1), 6.64 (2H, d, *J* 8.4), 3.75 (2H, s), 2.94 (2H, dd, *J* 7.2, *J* 7.6), 2.79 (2H, dd, *J* 7.2, *J* 7.6); ^{13}C NMR (100 MHz, DMSO-d_6): 176.3, 165.4, 162.9, 157.7, 156.2, 140.5, 139.9, 138.3, 130.7, 129.7, 129.3, 129.0, 127.6, 127.4, 127.0, 126.9, 119.7, 115.61, 115.56, 115.3, 102.4, 34.1, 32.0, 28.9. Anal. ($C_{30}H_{24}O_4$) C, H.

4.1.8.3. 7-Hydroxy-2-(4′-hydroxyphenylpropyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (6) (from 24c). Yield 75%; mp 166–8 °C; ^{1}H NMR (400 MHz, DMSO-d_6): 10.70 (1H, s), 9.15 (1H, s), 7.90 (1H, d, *J* 8.4), 7.60 (2H, d, *J* 7.6), 7.52 (2H, d, *J* 8.0), 7.43 (2H, t), 7.33 (1H, t, *J* 7.0), 7.20 (2H, d, *J* 8.0), 6.93 (2H, d, *J* 8.2), 6.91 (1H, dd, *J* 1.7, *J* 8.2), 6.82 (1H, d, *J* 1.7), 6.65 (2H, d, *J* 8.2), 3.80 (2H, s), 2.67 (2H, dd, *J* 7.4, *J* 7.7), 2.49 (2H, dd, *J* 7.4, *J* 7.7), 1.77 (2H, m); ^{13}C NMR (100 MHz, DMSO-d_6): 176.3, 166.3, 162.9, 157.7, 155.9, 140.5, 140.2, 138.3, 131.7, 129.6, 129.3, 129.0, 127.6, 127.4, 127.1, 126.9, 119.5, 115.6, 115.5, 115.2, 102.4, 34.3, 31.5, 29.3, 29.0. Anal. ($C_{31}H_{26}O_4$) C, H.

4.1.8.4. 6-Hydroxy-2-(4′-hydroxybenzyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (7) (from 24d). Yield 62%; mp 135–7 °C; ^{1}H NMR (DMSO-d_6): 9.90 (1H, broad s), 9.89 (1H, broad s), 9.22 (1H, broad s), 7.70–7.23 (11H, m), 7.17 (1H, dd, *J* 3.0, *J* 9.0), 7.00 (2H, d, *J* 8.5), 6.65 (2H, d, *J* 8.5), 3.99 (4H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 163.0, 159.8, 157.9, 157.4, 138.8, 138.2, 131.3, 130.6, 130.4, 128.6, 128.0, 127.4, 126.3, 123.9, 118.5, 116.1, 115.8, 115.6, 115.4, 102.6, 30.8. Anal. ($C_{29}H_{22}O_4$) C, H.

4.1.8.5. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(4″-hydroxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (8) (from 28a). Yield 71%; mp 183–5 °C (dec.); Ms (m/z): 436 [M$^+$]; ^{1}H NMR (400 MHz, DMSO-d_6): 10.74 (1 H, broad s), 10.07 (1H, broad s), 9.48 (1H, broad s), 7.89 (1H, d, *J* 8.7), 7.47 (2H, d, *J* 8.6), 7.43 (2H, d, *J* 8.1), 7.42 (2H, d, *J* 8.5), 7.10 (2H, d, *J* 8.1), 6.97–6.84 (4H, m), 6.82 (2H, d, *J* 8.5), 3.85 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 163.0, 159.8, 157.9, 157.4, 138.8, 138.2, 131.3, 130.6, 130.4, 128.6, 128.0, 127.4, 126.3, 123.9, 118.5, 116.1, 115.8, 115.6, 115.4, 102.6, 30.8. Anal. ($C_{28}H_{20}O_5$) C, H.

4.1.8.6. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(3″-carboxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (9) (from 28b). Yield 66%; mp 280–3 °C (dec.); ^{1}H NMR (400 MHz, DMSO-d_6): 10.85 (1H, broad s), 10.09 (1H, broad s), 8.14 (1H, s), 7.94–7.84 (3H, m), 7.62–7.43 (5H, m), 7.28–7.15 (2H, m), 6.98–6.83 (4H, m), 3.89 (2 H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 167.7, 163.1, 162.7, 159.9, 157.9, 140.6, 137.3, 131.9, 131.3, 130.6, 129.7, 128.9, 128.5, 127.5, 127.4, 127.2, 123.9, 118.6, 118.3, 116.6, 115.8, 115.6, 102.6, 30.9. Anal. ($C_{29}H_{20}O_6$) C, H.

4.1.8.7. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(3″,4″-dihydroxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (10) (from 28c). Yield 65%; mp 212–15 °C; Ms (*m*/*z*): 452 [M$^+$]; ^{1}H NMR (400 MHz, DMSO-d_6): 10.80 (1H, broad s), 10.05 (1H, broad s), 7.89 (1H, d, *J* 8.7), 7.46 (2H, d, *J* 8.5), 7.37 (2 H, d, *J* 7.7), 7.09 (2H, d, *J* 7.7), 6.98 (1H, s), 6.96–6.84 (5H, m), 6.78 (1H, d, *J* 8.2), 3.86 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 163.0, 162.6, 159.8, 157.9, 146.0, 145.4, 138.8, 138.5, 131.9, 130.6, 128.6, 127.4, 126.3, 123.9, 118.4, 117.9, 116.5, 115.8, 115.6, 115.4, 114.2, 102.6, 30.8. Anal. ($C_{28}H_{20}O_6$) C, H.

4.1.8.8. 2-(4′-Hydroxyphenyl)-3-[(3″,4″-dihydroxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (11) (from 28g). Yield 49%; mp 250–2 °C (Methanol); ^{1}H NMR (400 MHz, DMSO-d_6): 10.20 (1H, broad s), 9.00 (1H, broad s), 8.06 (1H, dd, *J* 1.3, *J* 7.9), 7.81 (1H, m), 7.68 (1H, d, *J* 8.3), 7.52 (2H, d, *J* 8.7), 7.56–7.46 (1H, m), 7.38 (2H, d, *J* 8.2), 7.10 (2H, d, *J* 8.2), 6.99 (1H, d, *J* 2.0), 6.91 (2H, d, *J* 8.7), 6.88 (1H, d, *J* 2.0, *J* 8.2), 6.78 (1H, d, *J* 8.2), 3.90 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 177.5, 163.3, 160.1, 156.1, 150.0, 145.4, 138.5, 134.5, 131.8, 130.7, 128.6, 126.3, 125.63, 125.56, 123.7, 122.7, 119.0, 118.8, 117.9, 116.5, 115.9, 114.2, 30.9. Anal. ($C_{28}H_{20}O_5$) C, H.

4.1.8.9. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(4″carboxy-3″-hydroxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (12) (from 28d). Yield 64%, mp > 280 °C; ^{1}H NMR (400 MHz, DMSO-d_6): 7.89 (1H, d, *J* 8.7), 7.59 (2H, d, *J* 8.6), 7.46 (2H, d, *J* 8.5), 7.21–7.12 (3H, m), 7.09 (1H, s), 6.88 (2H, d, *J* 8.5), 6.97–6.84 (3H, m), 3.89 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 164.0, 163.0, 162.7, 160.9, 159.9, 159.8, 157.9, 140.9, 137.3, 131.0, 130.6, 128.8, 127.3, 127.2, 123.9, 118.3, 118.2, 116.0, 115.9, 115.6, 115.4, 115.2, 102.6, 30.9. Anal. ($C_{29}H_{20}O_7$) C, H.

4.1.8.10. 2-(4′-Hydroxyphenyl)-3-[(4″carboxy-3″-hydroxy-1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (13) (from 28h). Yield 55%; mp 267–70 °C; Ms (Q-TOF Global Ultima, Micromass, Waters, MALDI, CH_3OH solution) (*m*/*z*) [M+H]$^+$: 465.11; ^{1}H NMR (400 MHz, DMSO-d_6): 10.26 (1H, broad s), 8.06 (1H, d, *J* 8.0), 7.82 (1H, m), 7.73 (1H, d, *J* 8.4), 7.68 (1H, d, *J* 8.4), 7.55–7.44 (5H, m), 7.15 (2H, d, *J* 8.3), 6.92 (2H, d, *J* 8.5), 6.92–6.84 (2H, m), 3.96 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 177.5, 172.2, 163.43, 163.37, 160.2, 156.1, 143.7, 139.8, 138.3, 134.5, 131.0, 130.7, 128.6, 127.1, 125.6, 125.5, 123.6, 122.7, 118.94, 118.87, 118.8, 115.9, 115.1, 114.0, 31.0. Anal. ($C_{29}H_{20}O_6$) C, H.

4.1.8.11. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(3″,4″-dihydroxy-1,1′-biphenyl-3-yl)methyl]-4*H*-1-benzopyran-4-one (14) (from 28e). Yield 66%; mp 138–40 °C (dec.); Ms (*m*/*z*): 452 [M$^+$]; ^{1}H NMR (400 MHz, DMSO-d_6): 10.83 (1H, broad s), 10.09 (1H, broad s), 9.00 (2H, broad s), 7.90 (1H, d, *J* 8.8), 7.48 (2H, d, *J* 8.6), 7.34–7.19 (4 H, m), 6.89 (2H, d, *J* 8.6), 7.02–6.74 (5H, m), 3.88 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 163.0, 162.6, 159.9, 157.9, 146.0, 145.6, 141.2, 140.9, 132.1, 130.6, 129.2, 127.4, 126.2, 125.9, 124.0, 118.5, 118.1, 116.5, 115.8, 115.6, 115.4, 114.3, 102.6, 31.2. Anal. ($C_{28}H_{20}O_6$) C, H.

4.1.8.12. 7-Hydroxy-2-(4′-hydroxyphenyl)-3-[(4″carboxy-3″-hydroxy-1,1′-biphenyl-3-yl)methyl]-4*H*-1-benzopyran-4-one (15) (from 28f). Yield 69%; mp >280 °C; ^{1}H NMR (400 MHz, DMSO-d_6): 10.81 (1H, broad s), 10.11 (1H, broad s), 7.90 (1H, d, *J* 8.8), 7.73 (1H, d *J* 8.4), 7.49 (2H, d, *J* 8.6), 7.39 (1H, d, *J* 7.9), 7.34–7.24 (2H, m), 7.03 (1H, d, *J* 8.0), 6.90 (2H, d, *J* 8.6), 6.96–6.77 (4H, m), 3.90 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 172.2, 163.4, 162.9, 162.4, 159.9, 158.1, 144.0, 141.3, 140.6, 131.0, 130.6, 129.2, 127.32, 127.30, 126.7, 124.9, 123.9, 119.8, 118.6, 115.9, 115.6, 115.3, 115.2, 114.2, 102.6, 31.1. Anal. ($C_{29}H_{20}O_7$) C, H.

4.1.8.13. 6,7-Dihydroxy-2-(4′-hydroxyphenyl)-3-[(1,1′-biphenyl-4-yl)methyl]-4*H*-1-benzopyran-4-one (16) (from 27a). Yield 44%; mp >280 °C; Ms (*m*/*z*): 436 [M$^+$]; ^{1}H NMR (400 MHz, DMSO-d_6): 7.63 (2H, d, *J* 7.4), 7.54 (2H, d, *J* 8.2), 7.49–7.39 (4H, m), 7.33 (1H, t, *J* 7.4), 7.15 (2H, d, *J* 8.2), 7.04 (1H, s), 6.88 (2H, d, *J* 8.9), 6.79 (1H, s), 3.89 (2H, s); ^{13}C NMR (100 MHz, DMSO-d_6): 176.7, 161.6, 159.6, 157.9, 152.5, 150.4, 140.6, 140.4, 138.1, 130.5, 129.3, 128.7, 127.6, 127.0, 126.9, 124.4, 117.6, 115.8, 115.3, 99.8, 97.3, 31.0. Anal. ($C_{28}H_{20}O_5$) C, H.

4.1.8.14. 3″,4′-Dihydroxy-1,1′-biphenyl (18) (from 30a). Yield 44%; mp 126–8 °C (137–9 °C[37]); Ms (*m/z*): 186 [M^+]; ^{1}H NMR ($CDCl_3$): 7.55–7.46 (2H, m), 7.43–7.22 (3H, m), 7.02 (1H, d, *J* 2.2), 6.92 (1H, dd, *J* 2.2, *J* 8.2), 6.80 (1H, d, *J* 8.2). Anal. ($C_{12}H_{10}O_2$) C, H.

4.1.8.15. 4′-Carboxy-3′-Hydroxy-1,1′-biphenyl (19) (from 30b). Yield 56%; mp 202–4 °C (207–8 °C[38]); ^{1}H NMR (DMSO-d_6): 7.82 (1H, d, *J* 8.0), 7.70 (2H, d, *J* 7.7), 7.56–7.32 (3H, m), 7.24–7.08 (2H, m). Anal. ($C_{13}H_{10}O_3$) C, H.

4.1.8.16. 2′-4′-Dihydroxy-1,1′-biphenyl (20) (from 30c). Yield 65%; mp 127–9 °C (145 °C[39]); Ms (*m/z*): 186 [M^+]; ^{1}H NMR ($CDCl_3$): 7.60–7.30 (5H, m), 7.10 (1H, d, *J* 9.2), 6.50 (2H, m). Anal. ($C_{12}H_{10}O_2$) C, H.

4.2. Molecular modeling

All protein alignments, protein and ligand preparations, and docking studies were performed using the Schrödinger Suite 2007, including MAESTRO 8.0, GLIDE 4.5, and PRIME 1.6,[25] on a PC equipped with a 2.13 GHz Core 2 Duo processor and 3 GB of RAM, running Fedora Core 8 Linux. Figure 1 was created with PYMOL v0.99.[40] Pharmacophore feature detection was performed with LIGANDSCOUT v2.01,[41] which was also used to create the graphics in Figure 2.

4.3. Enzyme section

The complete coding sequences of human LMW-PTP (IF1 and IF2 isoforms) and human PTP-1B were cloned in frame with the sequence of the glutathione *S*-transferase (GST) in the p-GEX-2T bacterial expression vector. Enzyme expression and purification were achieved in the *E. coli* TB1 strain.[42] Briefly, the recombinant fusion proteins were purified from bacterial lysate using a single-step affinity chromatography. The solution containing purified fusion proteins was treated with thrombin for 3 h at 37 °C. Then the enzymes were purified from GST and thrombin by gel filtration. The purity of proteins preparations was analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli.[43]

(a) Enzymatic assay and inihibition experiments. The enzymatic assay was carried out at 37 °C using *p*-nitrophenylphosphate as substrate; the final volume was 1 mL. The assay buffer (pH 7.0) contained 0.075 M of β,β-dimethylglutarate buffer, 1 mM EDTA and 5 mM dithiothreitol. The reactions were initiated by addition of aliquots of the enzyme preparations and stopped at appropriate times by adding 4 mL of 1 M KOH. The released p-nitrophenol was determined by reading the absorbance at 400 nm (ε = 18,000 M^{-1} cm^{-1}). K_m and V_{max} were determined by measuring the initial rates at different substrate concentrations. Experimental data were analysed using the Michaelis equation and a nonlinear fitting program (FIGSYS).

Inhibition constants were determined measuring initial hydrolysis rates at differing substrate and inhibitor concentrations. The apparent K_m values measured at the various inhibitor concentrations were plotted against concentration of the inhibitor to calculate the K_i values. All initial rate measurements were carried out in triplicate. For each inhibitor, IC_{50} was determined by measuring the initial hydrolysis rate under fixed *p*-nitrophenylphosphate concentration, equal to the K_m value of the considered PTP. Data were fitted to the following equation using the FIGSYS program: $V_i/V^\circ = IC_{50}/IC_{50} + [I])$, where V_i is the reaction velocity when the inhibitor concentration is [I], V° is the reaction velocity with no inhibitor and $IC_{50} = K_i + K_i[S]/K_m$. Therefore, when the substrate concentrations [*S*] is equal to K_m, $IC_{50} = 2K_i$.

(b) Cell-based Assays. HepG2 cells were routinely cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum in a 5% CO_2 humidified atmosphere. Subconfluent cells were serum-starved for 20 h before receiving inhibitors or insulin. Inhibitors ((10 μM final concentration) or insulin (10 nM final concentration) were added to the culture medium and the cells were incubated for 90 min at 37 °C. Cells were then lysed with SDS Laemmly sample buffer and analyzed by SDS-PAGE and Western Blot (anti-phosphorylated insulin receptor, anti-Akt and anti-actin antibodies were purchased from Santa Cruz Biotechnology Inc.; monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc.).

Acknowledgement

Work supported in part by the Italian MIUR (FIRB-2003 project RBNE03FMCJ_007).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.060.

References and notes

1. Fischer, E. H. *Adv. Enzyme Regul.* **1999**, *39*, 359.
2. Hubbard, S. R.; Till, J. H. *Annu. Rev. Biochem.* **2000**, *69*, 373.
3. Hunter, T. *Cell* **2000**, *100*, 113.
4. Neel, B. G.; Tonks, N. K. *Curr. Opin. Cell Biol.* **1997**, *9*, 193.
5. Andersen, J. N.; Mortensen, O. H.; Peters, G. H.; Drake, P. G.; Iversen, L. F.; Olsen, O. H.; Jansen, P. G.; Andersen, H. S.; Tonks, N. K.; Moller, N. P. *Mol. Cell Biol.* **2001**, *21*, 7117.
6. Caselli, A.; Marzocchini, R.; Camici, G.; Manao, G.; Moneti, G.; Pieraccini, G.; Ramponi, G. *J. Biol. Chem.* **1998**, *273*, 32554.
7. Wo, Y. Y. P.; McCormack, A. L.; Shabanowitz, J.; Hunt, D. F.; Davis, J. P.; Mitchell, G. L.; Van Etten, R. L. *J. Biol. Chem.* **1992**, *267*, 10856.
8. Chiarugi, P.; Taddei, M. L.; Schiavone, N.; Papucci, L.; Giannoni, E.; Fiaschi, T.; Capaccioli, S.; Raugei, G.; Ramponi, G. *Oncogene* **2004**, *23*, 3905.
9. Kikawa, K. D.; Vidale, D. R.; van Etten, R. L.; Kinch, M. S. *J. Biol. Chem.* **2002**, *277*, 39274.
10. Malentacchi, F.; Marzocchini, R.; Gelmini, S.; Orlando, C.; Serio, M.; Ramponi, G.; Raugei, G. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 875.
11. Chiarugi, P.; Cirri, P.; Marra, F.; Raugei, G.; Camici, G.; Manao, G.; Ramponi, G. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 676.
12. Taddei, M. L.; Chiarugi, P.; Cirri, P.; Talini, D.; Camici, G.; Manao, G.; Raugei, G.; Ramponi, G. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 564.
13. Sarabu, R.; Tilley, J. *Ann. Rep. Med. Chem.* **2005**, *40*, 167.
14. Zhang, M.; Stauffacher, C. V.; Lin, D.; Van Etten, R. L. *J. Biol. Chem.* **1998**, *273*, 21714.
15. Bleasdale, J. E.; Ogg, D.; Palazuk, B. J.; Jacob, C. S.; Swanson, M. L.; Wang, X. Y.; Thompson, D. P.; Conradi, R. A.; Mathews, W. R.; Laborde, A. L.; Stuchly, C. W.; Heijbel, A.; Bergdahl, K.; Bannow, C. A.; Smith, C. W.; Svensson, C.; Liljebris, C.; Schostarez, H. J.; May, P. D.; Stevens, F. C.; Larsen, S. D. *Biochemistry* **2001**, *40*, 5642.
16. Maccari, R.; Paoli, P.; Ottanà, R.; Jacomelli, M.; Ciurlo, R.; Manao, G.; Steindl, T.; Langer, T.; Vigorita, M. G.; Camici, G. *Bioorg. Med. Chem.* **2007**, *15*, 5137.
17. Vidal, D.; Blobel, J.; Perez, Y.; Thormann, M.; Pons, M. *Eur. J. Med. Chem.* **2007**, *42*, 1102.
18. Miranda, M. A.; Okamoto, A. K.; Ferreira, C. V.; Silva, T. L.; Granjeiro, J. M.; Aoyama, H. *J. Enzyme Inhib. Med. Chem.* **2006**, *21*, 419.
19. Wang, S.; Tabernero, L.; Zhang, M.; Harms, E.; Van Etten, R. L.; Stauffacher, C. V. *Biochemistry* **2000**, *39*, 1903.
20. Zabell, A. P. R.; Corden, S.; Helquist, P.; Stauffacher, C. V.; Wiest, O. *Bioorg. Med. Chem.* **2004**, *12*, 1867.
21. Ferrell, J. E.; Chang Sing, P. D.; Loew, G.; King, R.; Mansour, J. M.; Mansour, T. E. *Mol. Pharmacol.* **1979**, *16*, 556.
22. Wang, S.; Stauffacher, C. V.; Van Etten, R. L. *Biochemistry* **2000**, *39*, 1234.
23. Chen, R. M.; Hu, L. H.; An, T. Y.; Li, J.; Shen, Q. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3387.
24. Costantino, L.; Rastelli, G.; Gamberini, M. C.; Vinson, J. A.; Bose, P.; Iannone, A.; Staffieri, M.; Antolini, L.; Del Corso, A.; Mura, U.; Albasini, A. *J. Med. Chem.* **1999**, *42*, 1881.
25. Schrödinger Suite 2007; Schrödinger, LLC; New York, NY, USA, 2007.
26. Malamas, M. S.; Sredy, J.; Moxham, C.; Katz, A.; Xu, W.; McDevitt, R.; Adebayo, F. O.; Sawicki, D. R.; Seestaller, L.; Sullivan, D.; Taylor, J. R. *J. Med. Chem.* **2000**, *43*, 1293.
27. Wolfbeis, O. S.; Leiner, M.; Hochmuth, P.; Geiger, H. *Ber. Bunsenges. Phys. Chem.* **1984**, *88*, 759.
28. Maccari, R.; Ottanà, R.; Ciurleo, R.; Rakowitz, D.; Matuszczak, B.; Laggner, C.; Langer, T. *Bioorg. Med. Chem.* **2008**, *16*, 5840.

29. Steuber, H.; Zentgraf, M.; Podjarny, A.; Heine, A.; Klebe, G. *J. Mol. Biol.* **2006**, *356*, 45.
30. (a) Said, H.; Karickhoff, S. W.; Carreira, L. A. *Quant. Struct.-Act. Relat.* **1995**, *14*, 348; (b) SPARC on line calculator v4.1, http://ibmlc2.chem.uga.edu/sparc/, accessed Jan. 2008.
31. Herrero-Martínez, J. M.; Sanmartin, M.; Rosés, M.; Bosch, E.; Ràfols, C. *Electrophoresis* **2005**, *26*, 1886.
32. Zabell, A. P.; Schroff, A. D.; Bain, B. E.; Van Etten, R. L.; Wiest, O.; Stauffacher, C. V. *J. Biol. Chem.* **2006**, *281*, 6520.
33. Sherman, W.; Day, T.; Jacobson, M. P.; Friesner, R. A.; Farid, R. *J. Med. Chem.* **2006**, *49*, 534.
34. Zhang, M.; Zhou, M.; Van Etten, R. L.; Stauffacher, C. V. *Biochemistry* **1997**, *36*, 15.
35. Zhao, P. L.; Liu, C. L.; Huang, W.; Wang, Y. Z.; Yang, G. F. *J. Agric. Food Chem.* **2007**, *55*, 5697.
36. Bargellini, G.; Monti, L. *Gazz. Chim. Ital.* **1914**, *44*, 25.
37. Lin, P. H.; Sangaiah, R.; Ranasinghe, A.; Ball, L. M.; Swenberg, J. A.; Gold, A. *Org. Biomol. Chem.* **2004**, *2*, 2624.
38. Walker, J. *J. Chem. Soc.* **1939**, 120.
39. Suter, C. M.; Perrin, G. S. *J. Am. Chem. Soc.* **1939**, *61*, 166.
40. PYMOL Molecular Graphics System v0.99, DeLano Scientific, Palo Alto, CA, USA, 2006.
41. (a) Wolber, G.; Dornhofer, A.; Langer, T. *J. Comput. Aided Mol. Des.* **2006**, *20*, 773; (b) Wolber, G.; Langer, T. *J. Chem. Inf. Comput. Sci.* **2005**, *45*, 160–169; (c) LIGANDSCOUT 2.0; Inte:Ligand GmbH, Vienna, Austria, 2008.
42. Marzocchini, R.; Bucciantini, M.; Stefani, M.; Taddei, N.; Thunnissen, M. G. M. M.; Nordlund, P.; Ramponi, G. *FEBS Lett.* **1998**, *426*, 52.
43. Laemmli, U. K. *Nature* **1970**, *227*, 680.