European Journal of Medicinal Chemistry 48 (2012) 275–283

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and evaluation of platelet aggregation inhibitory activity of some 3-phenyl-pyrroloquinazolinones

Maria Grazia Ferlin [a,*], Christian Borgo [b], Renzo Deana [b]

[a] Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Padova, Via Marzolo 5, 35131 Padova, Italy
[b] Department of Biological Chemistry, University of Padova, Viale G. Colombo 3, 35131 Padova, Italy

ARTICLE INFO

Article history:
Received 6 July 2011
Received in revised form
14 December 2011
Accepted 16 December 2011
Available online 23 December 2011

Keywords:
Phenyl-pyrroloquinazolinones
Synthetic methods
Platelet aggregation
Cytosolic Ca^{2+} concentration
Tyrosine phosphorylation

ABSTRACT

A series of 3-phenyl-2,7-dihydro-1*H*-pyrrolo[3,2-*f*]quinazolin-1-one derivatives (3-PPyQZ) was synthesized starting from 5-amino-indoles, via condensation with *N*-ethoxycarbonylthiobenzamides followed by thermal cyclization. On the basis of their structural analogy with reported anti-thrombin pyrroloquinazolines, the derivatives were first tested for their capacity to inhibit platelet aggregation. Some of them had *in vitro* inhibitory effects on collagen and thrombin-induced aggregation in the micromolar range, and much higher inhibition than that shown by some phenyl-pyrroloquinolinones. Experiments to determine the mechanism of action of the most potent inhibitor (compound **18**) indicated that it acts in at least two sites: one preceding the agonist-induced increase of cytosolic [Ca^{2+}], and one following this step of the platelet activation cascade. The compound also inhibited thrombin-evoked protein–Tyr–phosphorylation. Although it is premature to draw definitive conclusions, the present results indicate that 3-PPyQZ structure, with the quite potent inhibitor of platelet aggregation compound **18,** might constitute a starting point for the synthesis of potential anti-thrombosis agents.

1. Introduction

Heterocycle quinazoline (Fig. 1), a bicyclic fused benzene and pyrimidine structure, is known to be a very interesting and useful scaffold, as it can be suitably modified with a variety of chemicals in order to obtain pharmacologically active compounds [1]. Quinazolinones are products deriving from oxidation of quinazolines, and like the latter, have attracted much attention due to their wide range of pharmacological properties [2]. Among the quinazolinone structures (Fig. 1), the most frequently encountered in medicinal chemistry is that of 4(3*H*)-quinazolinone, which has turned out to be particularly important, both as a synthetic intermediate [3] and as a natural product [4]. Its derivatives have been reported to possess various biological applications including antimicrobial [5], analgesic and anti-inflammatory [6,7], anti-tubercular [8], anti-malaria [9] and anti-cancer [10] activities.

Pyrroloquinazolines and pyrroloquinazolinones (PyQZs), consisting of a pyrrole ring fused to the quinazoline or quinazolinone moiety, comprise different structures, (depending on the fused sides), both natural (e.g., vasicine and its keto-analog vasicinone [11], and the luotonine family [12]) and synthetic ones [13,14].

Recently, continuing a research line aiming at synthesizing and studying anticancer compounds [15], some 3-phenyl-2,7-dihydro-1*H*-pyrrolo[3,2-*f*]quinazolin-1-ones (3-PPyQZs) have been prepared in our laboratory as analogs of antimitotic 7-phenyl-pyrrolo[3,2-*f*]quinolin-9-ones (7-PPyQs) modified at position 8 by replacing CH with N (Fig. 1) [16]. The 3-PPyQZs assayed against a panel of human cancer cell lines did not show significant cytotoxicity (data not shown). As 3-PPyQZs share the tricyclic nucleus with some amino-PyQZs having anti-thrombin activity [17–19], we thought it would be interesting to evaluate their potential inhibitory activity on platelet aggregation. Inhibition of platelet aggregation in humans is predicted to be beneficial in thrombotic disorders, and safer and resistance-free new drugs are needed in this field. Four 3-PPyQZs were tested, one unsubstituted at pyrrolic N (**18**) and three bearing alkyl groups (**19**, **21** and **22**) at this position (Fig. 2). In this screening, we also included four previously described non-cytotoxic PPyQs **24**–**27** [15,20] belonging to the two isomeric [3,2-*f*] and [2,3-*h*] structures (Fig. 2). These derivatives were selected because they share the 3-phenyl-quinolin-4-one (3-PQ) moiety with some 3-PQs reported to have anti-platelet activity

Abbreviations: PPyQ, phenyl-pyrroloquinolinones; 3-PPyQZ, 3-phenyl-2,7-dihydro-1*H*-pyrrolo[3,2-*f*]quinazolin-1-ones; ACD, citric-anticoagulant; PMA, phorbol myristate acetate; PKC, protein kinase C; LDH, lactate dehydrogenase.

* Corresponding author. Tel.: +390498271603; fax: +390498275366.
E-mail address: mariagrazia.ferlin@unipd.it1 (M.G. Ferlin).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.12.026

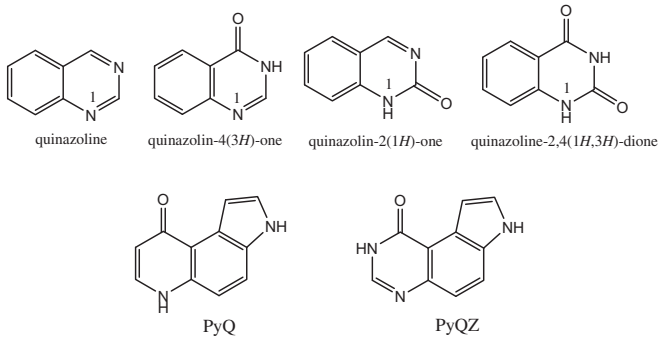


Fig. 1. Structures of heterocycles mentioned in the text.

[21]. We describe here the synthesis of these new 3-PPyQZs and preliminary results of a study assessing their anti-platelet activity and mechanism(s) of action.

2. Results and discussion

2.1. Chemistry

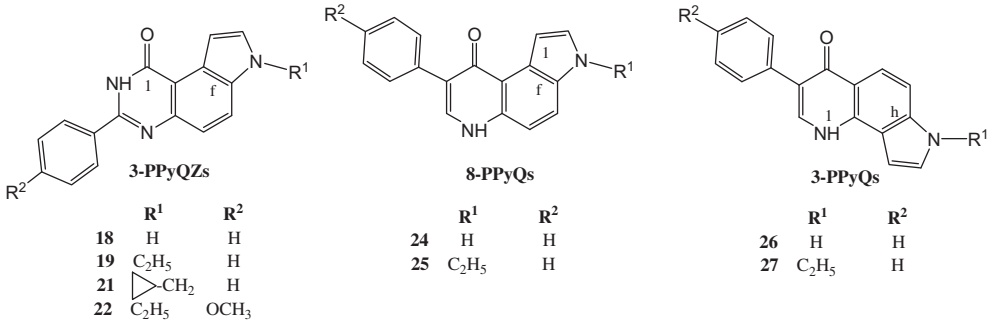
Two synthetic pathways leading to the pyrroloquinazoline molecular structure have been described: one, more complicated, exploits the well-established anthranilic acid pathway to quinazoline, and PyQZs are then obtained by various methods for constructing the indole nucleus [14]; the second starts from amino–indole and, via a condensation reaction with sodium dicyanamide, yields pyrrolequinazolines (Scheme 1) [22].

2.1.1. Synthesis

The new 3-PPyQZs were obtained from 5-amino-indole but applying another previously reported method for 3-PQZs [23,24]. First, N-substituted nitroindoles **2**–**4** (Scheme 2) were prepared by reaction with the adequate bromoalkane followed by catalytic hydrogenation to give the corresponding aminoindoles **5**–**8**, according to previously described procedures [16]. Aminoindole derivatives were then condensed with *N*-ethoxycarbonylthiobenzamides **11** and **12**. These were prepared in parallel by means of Friedel–Crafts thioacylation of **9** and **10** with ethoxycarbonyl isothiocyanate in the presence of anhydrous $AlCl_3$ [24]. The obtained *N*-indole-*N′*-ethoxycarbonylamidine compounds **13**–**17** were then cyclized in boiling diphenyl ether to final pyrroloquinazolinones **18**–**22** (Scheme 2A).

The general structure of the amidine intermediates **13**–**17** is supported by their IR spectra, which contain an absorption band at 1660–1680 cm^{-1} consistent with a conjugated carbonyl group (unsaturated α,β–amide), as well as ^1H NMR signals corresponding to the protons of the three aromatic rings and their substituents.

The exact structure of the newly synthesized PPyQZs was confirmed by 1-D and 2-D experiments. The ^1H NMR ($CDCl_3$) spectrum of compound **19**, taken as a sample, showed a singlet at δ 12.31 which, by analogy with literature data, was assigned to the proton in the 2 position (2-HN). Confirmation of this inference and complete structure assignments were achieved by ^{13}C NMR, HMQC and HMBC experiments. HMBC experiments revealed correlations within a distance of 2–3 C–H bonds (Fig. 3A, blue arrows). Diagnostic HMBC correlations were observed between the proton signals at δ 4.36 (CH_3–CH_2–) and carbon resonances at δ 129.8 (8-C) and 133.1 (6a–C) and from the signal at δ 8.03 (6-H) and carbons at δ 123.7 (9a-C), 144.8 (4a-C). Further correlations were observed

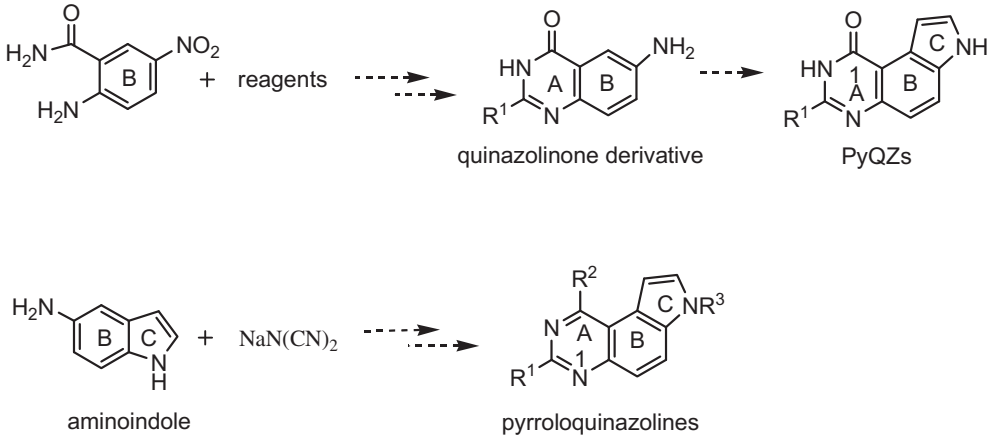


	R¹	R²
18	H	H
19	C_2H_5	H
21	▷CH_2	H
22	C_2H_5	OCH_3

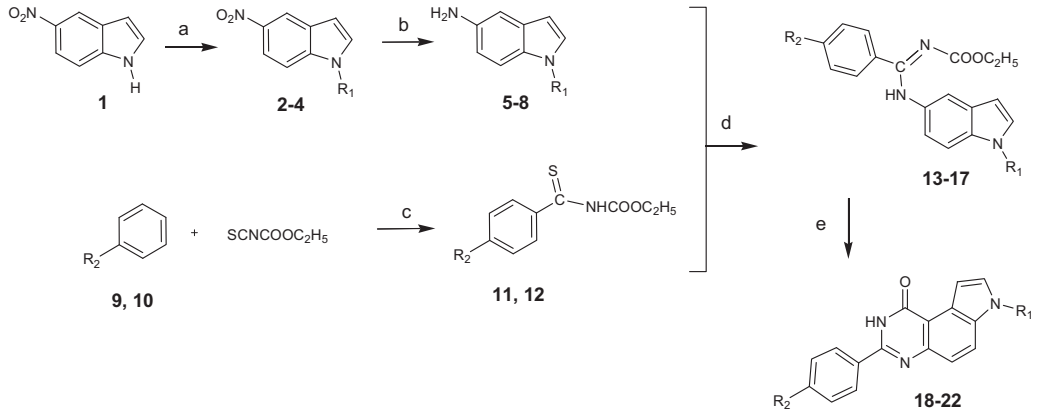
	R¹	R²
24	H	H
25	C_2H_5	H

	R¹	R²
26	H	H
27	C_2H_5	H

Fig. 2. Structures of tested compounds.



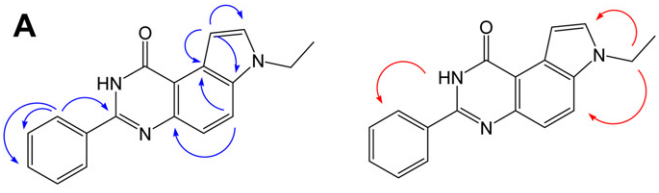
Scheme 1. Previously reported two pathways leading to pyrroloquinazoline derivatives.



compounds	R_1	R_2
5, 13, 18	H	H
2, 6, 14, 19	C_2H_5	H
3, 7, 15, 20	C_3H_7	H
4, 8, 16, 21	CH_2-cyclopropyl	H
9, 11	-‴	H
10, 12	-‴	OCH_3
17, 22	C_2H_5	OCH_3

Scheme 2. Synthetic route to phenyl-pyrroloquinazolinone derivatives **18**–**22**.

from the signal at δ 7.32 (9-H) and carbon at δ 117.5 (6-C). HMBC correlations from 2′- and 6′-H of the side phenyl ring (δ 8.22) were observed with carbon at δ 149.6 (3-C). In the NOESY spectrum (Fig. 3A, red arrows) correlations were observed between signal at δ 4.36 (CH_3–CH_2–) and proton signals at δ 7.64 and 8.03, confirming the aliphatic side-chain position and allowing the signal (*t*, $J = 3.1$ Hz) at δ 7.64 to be assigned to the proton in the 8 position and the doublet of doublets ($J = 8.8$ and 0.6 Hz) at δ 8.03 to the proton in the 6 position. In addition, the NOESY spectrum showed only one correlation between the signal at δ 12.31 (2-HN) and the multiplet at δ 8.22 (integral two protons), due to the 2′- and 6′-H of the side phenyl ring in the 3 position (Fig. 3B). In the case of the tautomer in which the proton is on the 4-N, two NOESY correlations should be observed, one between 4-H and the 2- and 6-H of the side phenyl ring, and the other between 4-H and 5-H.



7-ethyl-3-phenyl-2,7-dihydro-1*H*-pyrrolo[3,2-*f*]quinazolin-1-one **(19)**

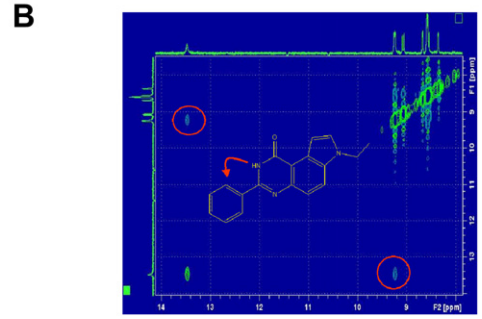


Fig. 3. (A) HMBC (blue arrows) and NOESY (red arrows) main correlations for compound **19**; (B) NOESY correlations at aromatic zone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Biology

The effect of 3-PPyQZs **18**–**22** and PPyQs **24**–**27** on platelet aggregation induced by thrombin is shown in Fig. 4. Compound **20** was not included in the analysed list because of its poor solubility in the test solvent. The concentration of the PPyQZs (8 μM) was chosen after some preliminary experiments with a range of various PPyQZ concentrations. Fig. 4 shows that, in the experimental conditions adopted, compound **18** caused complete inhibition of thrombin-induced platelet aggregation, whereas compounds **19**, **21** and **22** displayed lower inhibitory action and PPyQs very little inhibition on this process. Similar results were also obtained when the platelets were stimulated with collagen (data not shown). Thrombin and collagen were selected as agonists, since they are considered two of the most important platelet-stimulating agents [26–28]. As **18** turned out to be the strongest inhibitor of platelet aggregation, it was chosen for the following experiments, to elucidate its mechanism of action. Fig. 5 shows that **18**, in the experimental conditions adopted, inhibited thrombin-induced platelet aggregation in a concentration-dependent manner, causing complete inhibition at a concentration above 4 μM with an IC_{50} of 1.79 ± 0.28 μM. Inhibition was only slightly less in aspirin-treated platelets, indicating that 3-PPyQZ had only a slight effect on the cyclo-oxygenase level.

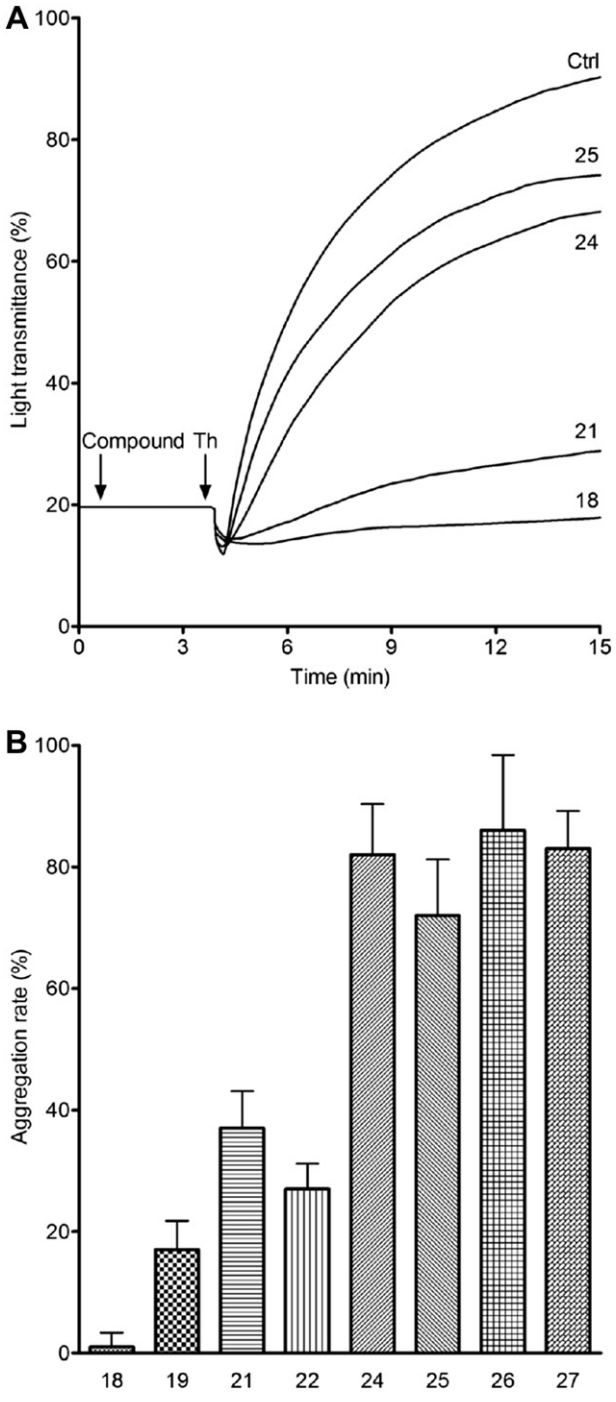


Fig. 4. Inhibitory effect of the new 3-PPyQZs **18**–**22** and the PPyQs **24**–**27** on thrombin-induced platelet aggregation. (A): representative traces of the experiments of platelet aggregation induced by thrombin (Th) (40 mU/mL), added at the arrow, 3 min after the 3-PPyQZs or PPyQ (8 μM) or vehicle (DMSO, Control) (B): initial aggregation rates (velocities) of aggregation induced by thrombin (40 mU/mL) added 3 min after the indicated compounds (8 μM). Results are means of four separate determinations with the S.E.M. indicated by vertical bars.

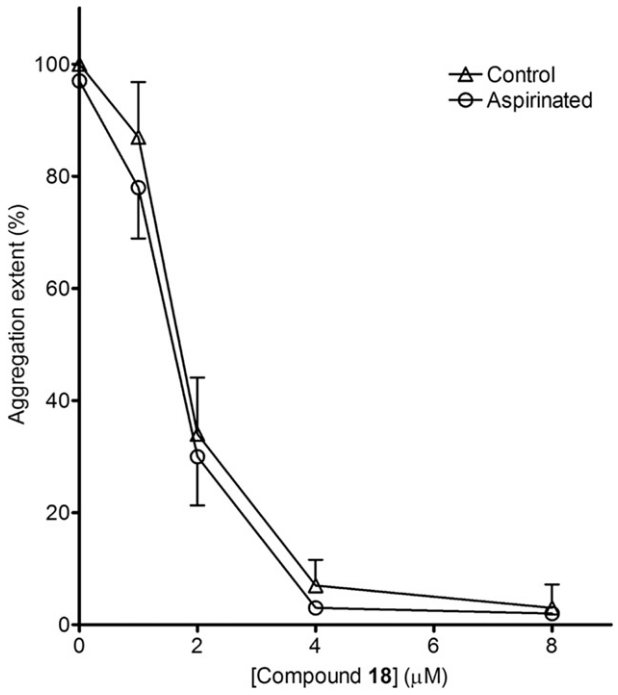


Fig 5. Inhibition of thrombin-induced platelet aggregation caused by increasing concentrations of compound **18**. Aggregation extent (magnitude) in untreated (Control) or aspirin-treated platelets, was measured 10 min after thrombin-addition (40 mU/mL), which was added at the reported concentrations 3 min after 3-PPyQZ **18**. Acetylsalicylic acid treatment was performed as described in the methods section. Data are means of 4 experiments with S.E.M. indicated by the vertical bars.

Platelet activation may also be caused by non-physiological agonists such as calcium ionophores, which give rise to an artificial increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), or protein-kinase C (PKC) activators, such as phorbol–esters [29]. The concentration-dependent inhibitory effect of **18** was also studied on platelet aggregation elicited by the non-physiological Ca^{2+}-ionophore ionomycin and the PKC activator phorbol–ester (PMA), as well as by collagen, another physiological agonist. The results reported in Fig. 6 show that the compound **18** inhibited the platelet aggregation promoted by collagen, on the one hand, slightly more efficiently than that promoted by thrombin (Fig. 5), and on the other hand, more strongly compared to that elicited by the artificial activators ionomycin or phorbol–ester. The latter results suggest that the compound acts at least at two sites: one preceding the agonist-induced cytosolic Ca^{2+} increase and protein-kinase activation and one following these events.

The action of **18** was then tested on the thrombin-induced $[Ca^{2+}]_c$ increase, which is considered as the pivotal step of the platelet activation cascade [26,30]. Compound **18** alone caused a slight increase in $[Ca^{2+}]_c$ and a large increment in the $[Ca^{2+}]_c$ rise produced by thrombin added 1 min after 3-PPyQZ (Fig. 7A). Instead, **18** caused a lowering of the $[Ca^{2+}]_c$ increase caused by thrombin added at least 3 min after the pyrroloquinazolinone (Fig. 7B).

In order to determine whether 3-PPyQZ exerted its action upstream of the thrombin-elicited intracellular Ca^{2+} movement, i.e., endoplasmic reticulum Ca^{2+} release, or on the subsequent entry of Ca^{2+} from the extracellular compartment [31], we carried out the above experiments with platelets suspended in a medium virtually devoid of Ca^{2+}, i.e., in the presence of the extracellular Ca^{2+}chelator EGTA. As expected under these conditions, the amplitude of the thrombin-induced $[Ca^{2+}]_c$ increase was less than that observed in the presence of external calcium (Fig. 8A and B), although the effects of **18** were substantially similar to those obtained with platelets suspended in a calcium-containing medium (Fig. 7A and B). These results indicate that 3-PPyQZ exerts its action mainly upstream of the intracellular Ca^{2+} movement from the endoplasmic reticulum to the cytosolic compartment.

As on one hand protein phosphorylation is an outstanding event associated with platelet activation [32–34] and, on the other, it has been reported that the anti-thrombotic properties of certain

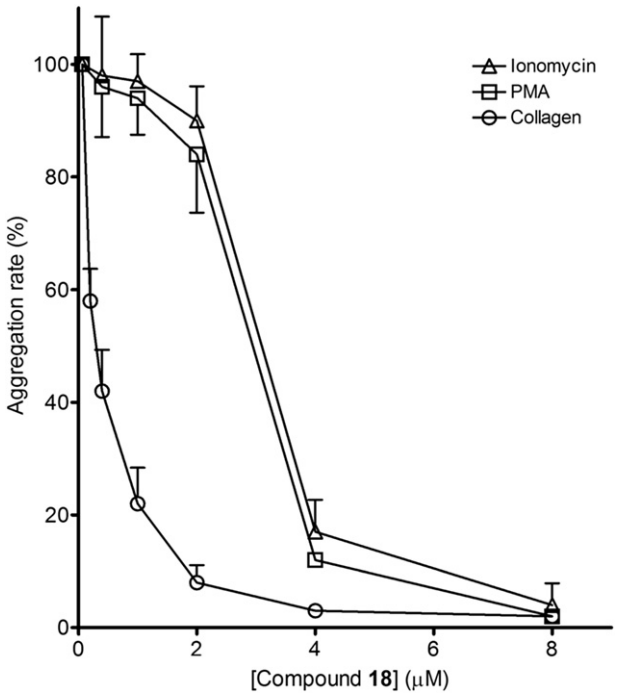


Fig. 6. Effect of **18** on platelet aggregation induced by collagen, or the calcium–ionophore ionomycin, or the PKC activator phorbol–ester (PMA). The indicated concentrations of **18** (or equivalent volume of the vehicle DMSO) were incubated for 3 min and then 40 μg/mL collagen, or 20 nM PMA or 0.5 μM ionomycin were added. The maximal aggregation rate measured after the agonist addition was considered 100% in the absence of 3-PPyQZ. Data are means of 4 experiments with S.E.M. indicated by the vertical bars.

pyrroloquinazolines are probably due to their inhibitory effect on protein–tyrosine–kinases [14,35], we investigated the effect of 3-PPyQZs on the levels of protein–tyrosine-phosphorylation in resting and thrombin-stimulated platelets. Fig. 9 shows that **18** added to resting platelets did not significantly alter the degree of tyrosine phosphorylation, but did produce a marked inhibition in the phosphorylation pattern obtained after thrombin stimulation. The inhibitory effect was particularly evident on proteins of about 58 and 80 kDa, the chemical nature of which is still undetermined. Lastly, we analysed the possible damaging effects of 3-PPyQZs on platelet membrane integrity by monitoring the release of lactate dehydrogenase (LDH) [36] from platelets incubated with the compound. No appreciable release of LDH was found after platelet incubation for 1 h with 4 μM **18** (data not shown). We also monitored the effect of this compound on platelet viability determined by the Alamar Blue method [37]. At the concentration used, **18** caused only a slight decrease in cellular viability after 4–6 h of incubation (not shown).

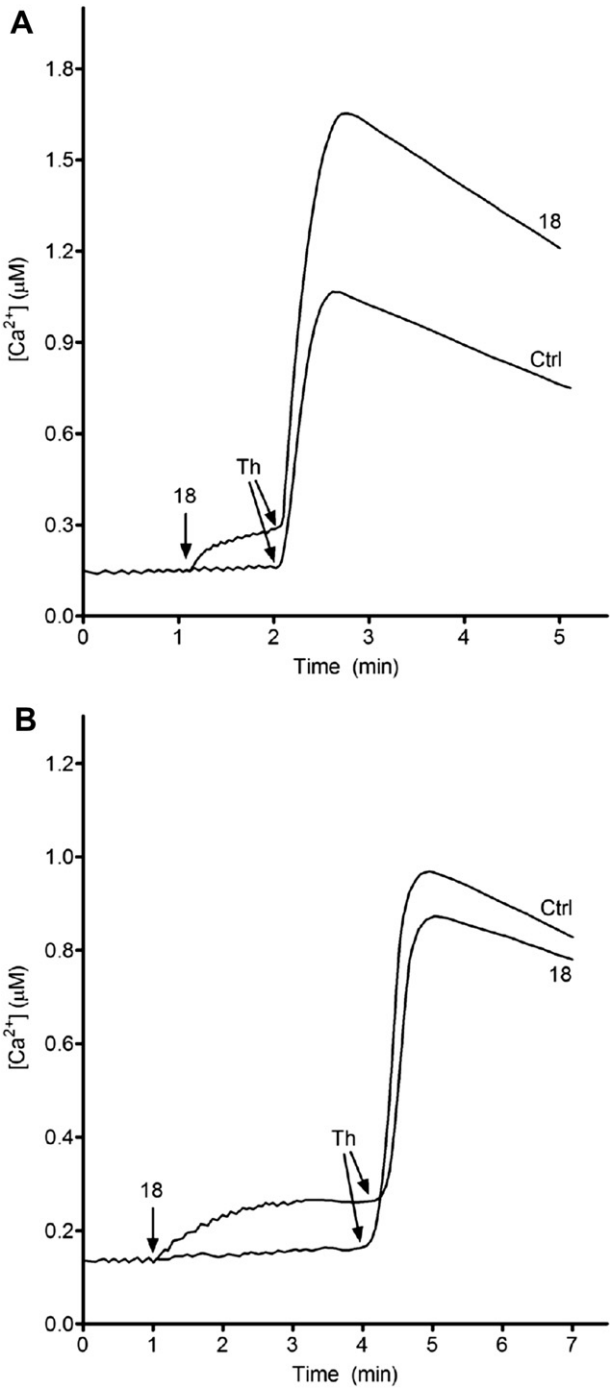


Fig. 7. Cytosolic [Ca^{2+}] changes produced by thrombin in platelets preincubated with vehicle or the 3-PPyQZ 18. (A): traces of [Ca2+]c increase induced by addition (at the arrow) of 40 mU/mL thrombin 1 min after the 3-PPyQZ **18** (2 μM) or an equivalent volume of DMSO (control). (B): changes of [Ca2+]c produced by thrombin added 3 min after **18**. Traces are representative of three separate experiments.

3. Conclusions

Two small series of tricyclic compounds, the newly synthesized PPyQz **18**–**22** and the previously reported PPyQ **24**–**27** derivatives were evaluated as anti-aggregation agents.

None of the tested PPyQ derivatives at the concentration adopted (8 μM), showed significant inhibitory effects on thrombin-induced platelet aggregation. This means that enlargement of the 3-phenyl–quinolinone model structure by fusing a pyrrole ring is detrimental for anti-platelet activity. At the same concentration (8 μM), 3-PPyQZs had interesting inhibitory effects, although to different extents. In general, it seems that the here made substitutions on the 3-PPyQZ scaffold are unfavorable for anti-platelet activity, as unsubstituted compound **18** is the most active. Regarding the alkyl substitutions at pyrrolic N, both ethyl and cycloprolylmethyl groups, cause a decrease of the inhibitory action of the compounds **19**, **22** and **21** in comparison with compound **18**, the decrease of inhibitory effect being greater for **21** with the bulky cyclopropylmethyl group (Fig. 4B). Moreover, the methoxylic group on the side phenyl ring causes a further decrease

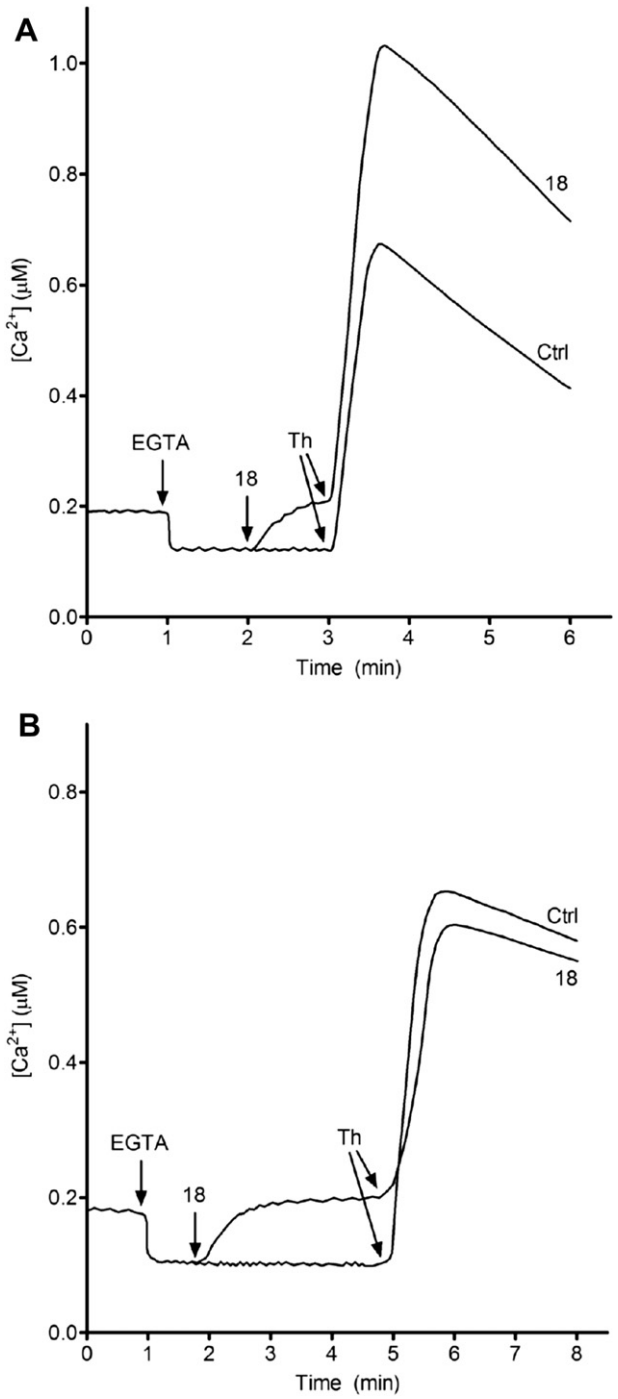


Fig. 8. Thrombin-induced [Ca2+]c increase in platelets pre-incubated with vehicle or the 3-PPyQZ **18** in the absence of extra-cellular calcium. (A) and (B): traces of [Ca2+]c increase induced by thrombin-addition (40 mU/mL at the arrow) 1 min or 3 min respectively after **18** (2 μM) which, in turn, was added 1 min after 0.5 mM EGTA. Traces are representative of three separate experiments.

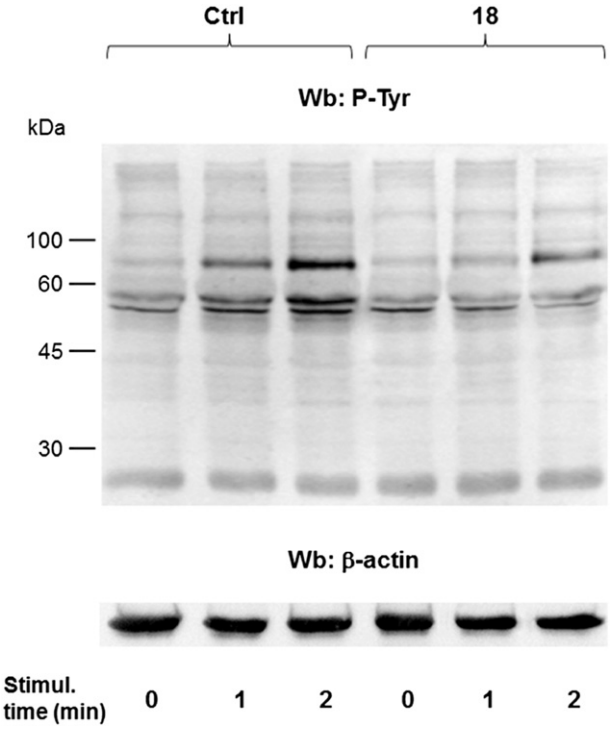


Fig. 9. Effect of **18** on protein tyrosine phosphorylation of resting and thrombin-stimulated platelets. Anti-P-Tyr immunostaining of proteins from platelets pre-treated with vehicle, (DMSO, control) or 2 μM 18 for 3 min and then stimulated with thrombin (40 mU/mL). Mass molecular markers were used for the determination of actinβ apparent molecular masses of the proteic bands. Anti immunoblotting was performed in parallel to control the amounts of proteins loaded in the SDS/PAGE lanes. Blots are representative of four experiments performed with different platelet samples.

in the inhibitory activity (**22** vs **19**). As **21** is the least active of all compounds, it is clear that aliphatic bulky groups at pyrrolic N should be avoided anyway (Fig. 4B).

However, in contrast with what has previously been described for some pyrroloquinazolines [18,38], compound **18** does not specifically interfere with the thrombin receptor PAR-1. In fact, the inhibitory effect of 3-PPyQZ is similar in platelet aggregation induced by PAR-interacting thrombin or PAR-independent collagen.

The finding that compound **18** inhibited the $[Ca^{2+}]_c$ increase induced by physiological agonists, even in the absence of extracellular calcium, indicates that it acts at a level preceding the agonist-induced increase in cytosolic $[Ca^{2+}]$ deriving from the endoplasmic reticulum. In addition, the inhibition displayed on aggregation elicited by compounds which caused direct increases in $[Ca^{2+}]_c$ or protein-kinase activity indicates that the compound also acts downstream of the agonist-induced increase in cytosolic $[Ca^{2+}]$. It is still difficult to establish whether the inhibitory effect caused by compound **18** on thrombin-elicited protein–Tyr phosphorylation is due to direct action on kinase/phosphatase enzymes or is a consequence of the reduced rise in $[Ca^{2+}]_c$. In conclusion, the present results indicate that 3-PPyQZ structure, with the quite potent inhibitor of platelet aggregation compound **18**, might constitute a starting point for the synthesis of potential anti-thrombosis agents.

4. Experimental

4.1. Materials and methods

Melting points were determined on a Gallenkamp MFB 595 010M/B capillary melting point apparatus, and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer 1760 FTIR spectrometer with potassium bromide pressed disks; all values are expressed in cm^{-1}. ^1H NMR spectra were determined on Bruker 300 and 400 MHz spectrometers, with the solvents indicated; chemical shifts are reported in δ (ppm) downfield from tetramethylsilane as internal reference. Coupling constants are given in Hertz. In the case of multiplets, chemical shifts were measured starting from the approximate center. Integrals were satisfactorily in line with those

expected on the basis of compound structure. Elemental analyses were performed in the Microanalytical Laboratory, Department of Pharmaceutical Sciences, University of Padova, on a Perkin–Elmer C, H, N elemental analyzer model 240B, and analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Mass spectra were obtained on a Mat 112 Varian Mat Bremen (70 ev) mass spectrometer and Applied Biosystems Mariner System 5220 LC/Ms (nozzle potential 250.00). Column flash chromatography was performed on Merck silica gels (250–400 mesh ASTM); chemical reactions were monitored by analytical thin-layer chromatography (TLC) on Merck silica gel 60 F-254 glass plates.

Solutions were concentrated on a rotary evaporator under reduced pressure. Starting materials were purchased from Aldrich Chimica and Acros, and solvents from Carlo Erba, Fluka and Lab-Scan.

Apyrase, prostacyclin, α-thrombin and sodium p-nitrophenyl-phosphate (pNPP) were purchased from Sigma Chemicals (St. Louis, MO) and fura 2/AM, ionomycin and sodium orthovanadate from Calbiochem (Darmstadt, Germany). Anti-phosphotyrosine monoclonal antibody PY20 was from ICN Biotechnology (Irvine, CA). All other reagents were of analytical grade.

4.1.1. Synthesis of N-ethoxycarbonylthiobenzamides (**11**)

To 15 mL of a stirred cold (ice bath) solution of CH_2Cl_2 containing 0.3901 g (d 0.88, 0.44 mL, 5 mmol) of benzene (**9**) and 0.6557 g (5 mmol) of ethoxycarbonyl isothiocyanate, was added 10 mmol of anhydrous $AlCl_3$ (1.3334 g) in small portions (10–15 min) at 0–3 °C. The reaction mixture was stirred at this temperature for 4 h (TLC ethyl acetate/*n*-hexane 8:2) and was then hydrolysed by careful addition of ice and 2 M hydrochloric acid. Volumes of CH_2Cl_2 sufficient to dissolve any solid organic material were added, and the resulting solution was extracted with four portions of 10% aqueous NaOH. This extract was washed with ethyl ether and acidified with concentrated hydrochloric acid (ice bath) to yield an oil, which solidified upon cooling. The solid material was then washed with 1 M hydrochloric acid and water, dried and washed again with cold aqueous ethanol (ethanol/water 6:4). The resulting dried solid product was used as such in the following reactions. Yield 69% [literature [25] 52%]; orange solid; mp 63–64 °C [literature [25] 61–61.5 °C], rf 0.69 (ethyl acetate/*n*-hexane 8:2); ^{1}H NMR (DMSO-d_6) δ 1.34 (*t*, 3H, *J* = 7.1 Hz, CH_2CH_3), 4.18 (*q*, 2H, *J* = 7.1 Hz, NCH_2CH_3), 7.36–7.64 (*m*, 5H, Ar), 12.18 (bs, 1H, S═C–*NH*); HRMS (ESI) $[M + H]^+$ calculated for $C_{10}H_{12}NO_2S$ *m*/*z* 210.0544; found 210.0559.

4.1.1.1. Synthesis of ethyl p-methoxy-phenyl-carbothionylcarbamate (**12**)

Compound **12** was prepared by the procedure of compound **11** by reaction of 0.6705 g (d 0.995, 0.674 mL, 6.2 mmol) and 1.643 g (12.4 mmol). Yield 88% [literature 90%]; orange yellowish solid; mp 90–91 °C [literature [26] 88–90 °C]; rf 0.77 (ethyl acetate/*n*-hexane 8:2); ^{1}H NMR (DMSO-d_6) δ 1.25 (*t*, 3H, *J* = 7.1 Hz, CH_2CH_3), 3.82 (*s*, 3H, OCH_3), 4.18 (*q*, 2H, *J* = 7.1 Hz, NCH_2CH_3), 6.93 (*d*, 2H, *J* = 8.8 Hz, Ar), 7.70 (*d*, 2H, *J* = 8.8 Hz, Ar.), 11.90 (bs, 1H, S═C–*NH*); HRMS (ESI) $[M + H]^+$ calculated for $C_{11}H_{13}NO_3S$ *m*/*z* 240.0650; found 240.0665.

4.1.2. Synthesis of N-indole-N′-ethoxycarbonylamidine derivative (**13**)

In a two-necked round-bottomed flask, a solution of amino-indole derivatives **5** [16] (2.6433 g, 20 mmol) and N-ethoxycarbonylthioamides **11** (2.0926 g, 10 mmol) in 30 mL of absolute ethanol was refluxed until hydrogen sulfite evolution had stopped (6–8 h), as indicated by lead acetate paper and TLC (ethyl acetate/*n*-hexane 8:2). At the end of the reaction, the solvent was evaporated under reduced pressure and the raw material was purified by silica gel Flash Chromatography (eluant ethyl acetate/*n*-hexane 8:2). Yield 40%; mp 151–155 °C; rf 0.76 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 1662 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 0.95 (*t*, 3H, *J* = 7.1 Hz; C(O)OCH$_2$*CH*$_3$), 3.82 (*q*, 2H, *J* = 7.1 Hz, C(O)O*CH*$_2$CH$_3$), 6.41–7.91 (*m*, 10H, Ar), 9.77 (*s*, 1H, N═C–*NH*), 11.06 (bs, 1H, *NH*).

4.1.2.1. Sinthesis of (E,Z)-ethyl(1-ethyl-1H-indol-5-ylamino)(phenyl) methylenecarbamate (**14**)

The synthesis of compound **14** was carried out by the same procedure as described for **13** by reaction of aminoindole derivative **6** (1.6022 g, 10 mmol) and **11** (2.0926 g, 5 mmol). Yield 50%; mp 148–150 °C; rf 0.80 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 1679 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 0.94 (*t*, 3H, *J* = 7.1 Hz; C(O)OCH$_2$*CH*$_3$,), 1.35 (*t*, 3H, *J* = 7.2 Hz, NCH$_2$*CH*$_3$), 3.82 (*q*, 2H, *J* = 7.1 Hz, C(O)O*CH*$_2$CH$_3$), 4.18 (*q*, 2H, *J* = 7.2 Hz; N*CH*$_2$CH$_3$), 6.41–7.92 (*m*, 10 H, Ar), 9.77 (bs, 1H, N═C–*NH*).

4.1.2.2. (E,Z)-Ethyl(1-propyl-1H-indol-5-ylamino)(phenyl) methylenecarbamate (**15**)

The synthesis of compound **15** was carried oput by the same procedure as described for **13** by reaction of aminoindole derivative **7** (1.4652 g, 8.4 mmol) and **11** (0.878 g, 4.2 mmol). Yield 46%; mp 149–150 °C; rf 0.85 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 1672 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 0.82 (*t*, 3H, *J* = 7.2 Hz, NCH$_2$CH$_2$*CH*$_3$), 0.94 (*t*, 3H, *J* = 7.1 Hz, C(O)OCH$_2$*CH*$_3$), 1.71–1.83 (*m*, 2H, NCH$_2$*CH*$_2$CH$_3$), 3.82 (*q*, 2H, *J* = 7.1 Hz, C(O)O*CH*$_2$CH$_3$), 4.11 (*t*, 2H, *J* = 7.2 Hz, N*CH*$_2$CH$_2$CH$_3$), 6.41–7.91 (*m*, 10H, Ar), 9.76 (bs, 1H, N═C–*NH*).

4.1.2.3. Synthesis of (E,Z)-ethyl(1-(cyclopropylmethyl)-1H-indol-5-ylamino)(phenyl)methylenecarbamate (**16**)

The synthesis of compound **16** was carried out by the same procedure as for **13** by reaction of aminoindole derivative **8** (1.2953 g, 6.95 mmol) and **11** (0.7272 g, 3.47 mmol). Yield 45%; mp 155–158 °C; rf 0.84 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 1675 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 0.37–0.52 (*m*, 4H, CH*CH*$_2$*CH*$_2$), 0.95 (*t*, 3H, *J* = 7.1 Hz, C(O)OCH$_2$*CH*$_3$), 1.17–1.28 (*m*, 1H, *CH*CH$_2$CH$_2$), 3.82 (*q*, 2H, *J* = 7.1 Hz, C(O)O*CH*$_2$CH$_3$), 4.02 (*d*, 2H, *J* = 7.2 Hz, N-*CH*$_2$,), 6.41–7.94 (m, 10H, Ar), 9.70 (bs, 1H, N═C–*NH*).

4.1.2.4. Synthesis of (E,Z)-Ethyl(1-ethyl-1H-indolo-5-ylamino-4-methoxy-phenyl)methylenecarbamate (**17**)

The synthesis of compound **17** was carried out by the same procedure as for **13** by reaction of aminoindole derivative **6** (1.9410 g, 12.11 mmol) and compound **12** 1.5554 g, 6.2 mmol. Yield 43%; mp 137–140 °C; rf 0.78 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 1667 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 1.00 (*t*, 3H, *J* = 7.2 Hz, NCH$_2$*CH*$_3$), 1.35 (*t*, 3H, *J* = 7.1 Hz, C(O)OCH$_2$*CH*$_3$), 3.81–3.85 (*m*, 5H, O*CH*$_3$ and C(O)O*CH*$_2$CH$_3$), 4.18 (*q*, 2H, *J* = 7.2 Hz, N*CH*$_2$CH$_3$), 6.40–7.86 (*m*, 9H, Ar), 9.70 (bs, 1H, N═C–*NH*).

4.1.3. Synthesis of 3-phenyl-2,7-dihydro-1H-pyrrolo[3,2-f] quinazolin-1-one (**18**)

In a two-necked, 50-mL, round-bottomed flask, 10–15 mL of diphenyl ether were heated to boiling temperature; 0.3326 g (1.08 mmol) of amidine **13** were then added portion-wise and the mixture was refluxed for 15–20 min. After cooling to 50 °C, the separated precipitate was collected by filtration and washed many times with diethyl ether. The collected product was purified by flash chromatography (ethyl acetate/*n*-hexane 8:2). Yield 54%, brown solid; mp >300 °C; rf 0.79 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 3227 (NH), 3217 (NH), 1690 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 7.33 (td, 1H, *J* = 2.7 and 0.8 Hz, 9H), 7.49 (*d*, 1H, *J* = 8.6 Hz, 5–H), 7.54 (*m*, 3H, *J* = 7.9 and *J* = 1.9 Hz, 3′-H-, 4′-H-, 5′-H), 7.59 (*t*, 1H, *J* = 2.8 Hz, 8-H), 7.89 (dd, 1H, *J* = 0.76 and

J = 8.6 Hz, 6-H), 8.20 (*m*, 2H, 2′-H and 6'-H), 11.69 (*s*, 1H, *NH* pyrrole), 12.39 (bs, 1H, *NHC*(O)); ^{13}C NMR (DMSO-d_6) δ 102.9, 112.7, 117.5, 120.6, 123.7, 127.5, 128.6, 129.8, 130.8, 133.0, 133.1, 144.9, 149.6, 162.4; HRMS (ESI) $[M + H]^+$ calculated for $C_{16}H_{12}N_3O$ *m*/*z* 262.0936; found 262.0948; anal. calcd. for $C_{16}H_{11}N_3O$: C, H, N.

4.1.3.1. Synthesis 7-ethyl-3-phenyl-2,7-dihydro-1H-pyrrolo[3,2-f] quinazolin-1-one (**19**)

The synthesis of compound **19** was carried out by the same procedure as for **18** by thermal cyclization of amidine derivative **14** (0.4450 g, 1.33 mmol). Yield 47%; light yellow solid; mp>300 °C; rf 0.72 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 3207 (NH), 1683 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.41 (*t*, 3H, J = 7.2 Hz, NCH_2CH_3,), 4.36 (*q*, 2H, J = 7.2 Hz, NCH_2CH_3), 7.32 (dd, 1H, J = 3.1 and J = 0.6 Hz, 9-H), 7.51 (*d*, 1H, J = 8.8 Hz, 5-H), 7.54 (*m*, 3H, 3′-H, 4′-H, 5′-H), 7.64 (*d*, 1H, J = 3.6 Hz, 8-H), 8.03 (dd, 1H, J = 8.8 and J = 0.6 Hz, 6-H), 8.22 (m, 2H, 2′-H and 6'-H), 12.31 (bs, 1H, *NHC*(O)); ^{13}C NMR (DMSO-d_6) δ 15.8, 40.6, 102.9, 112.7, 117.5, 120.6, 123.7, 127.5, 128.6, 129.8, 130.8, 133.0, 133.1, 144.8, 149.6, 162.4; HRMS (ESI) $[M + H]^+$ calculated for $C_{18}H_{16}N_3O$ *m*/*z* 290.1249; found 290.1262; anal. calcd. for $C_{18}H_{15}N_3O$: C, H, N.

4.1.3.2. Synthesis of 7-propyl-3-phenyl-2,7-dihydro-1H-pyrrolo [3,2-f]quinazolin-1-one (**20**)

The synthesis of compound **20** was carried out by the same procedure as for **18** by thermal cyclization of amidine derivative **15** (0.3493 g, 1.00 mmol). Yield 62%, white solid; mp 295-96 °C; rf 0.81 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 3212 (NH), 1671 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.85 (*t*, 3H, J = 7.2 Hz, $NCH_2CH_2CH_3$), 1.76–1.88 (*m*, 2H, $NCH_2CH_2CH_3$), 4.28 (*t*, 2H, J = 7.0 Hz, $NCH_2CH_2CH_3$), 7.32 (dd, 1H, J = 3.1 and J = 0.6 Hz, 9-H), 7.50 (*d*, 1H, J = 8.8 Hz, 5-H), 7.54 (*m*, 3H, 3′-H, 4′-H, 5′-H), 7.61 (*d*, 1H, J = 3.1 Hz, 8-H), 8.03 (dd, 1H, J = 8.8 and J = 0.6 Hz, 6-H), 8.22 (m, 2H, 2′-H and 6'-H), 12.42 (bs, 1H, *NHC*(O)); ^{13}C NMR (DMSO-d_6) δ 11.5, 33.4, 42.7, 102.9, 112.7, 117.5, 120.6, 123.7, 127.5, 128.6, 129.8, 130.8, 133.0, 133.1, 144.9, 149.6, 162.4; HRMS (ESI) $[M + H]^+$ calculated for $C_{19}H_{18}N_3O$ *m*/*z* 304.1405; found 304.1412; anal. calcd. for $C_{19}H_{17}N_3O$:C, H, N.

4.1.3.3. Synthesis of 7-cyclopropylmethyl-3-phenyl-2,7-dihydro-1H-pyrrolo[3,2-f]quinazolin-1-one (**21**)

The synthesis of compound **21** was carried out by the same procedure as for **18** by thermal cyclization of amidine derivative **16** (0.4802 g, 1.33 mmol). Yield 61%, brown solid; mp>300 °C; rf 0.74 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 3237 (NH), 1665 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.40–0.56 (*m*, 4H, $CHCH_2CH_2$), 1.23–1.31 (*m*, 1H, $CHCH_2CH_2$), 4.19 (*d*, 2H, J = 7.0 Hz, N-CH_2), 7.33 (dd, 1H, J = 3.1 and J = 0.6 Hz, 9-H), 7.50 (*d*, 1H, J = 8.8 Hz, 5-H), 7.54 (*m*, 3H, 3′-H, 4′-H, 5′-H), 7.68 (*d*, 1H, J = 3.1 Hz, 8-H), 8.10 (dd, 1H, J = 8.8 and J = 0.6 Hz, 6-H), 8.21 (m, 2H, 2′-H and 6′-H), 12.44 (bs, 1H, *NHC*(O)); ^{13}C NMR (DMSO-d_6) δ 4.10, 12.22, 50.22, 102.9, 112.7, 117.5, 120.6, 123.7, 127.5, 128.6, 129.8, 130.8, 133.0, 133.1, 144.9, 149.6, 162.4; HRMS (ESI) $[M + H]^+$ calculated for $C_{20}H_{18}N_3O$ *m*/*z* 316.1405; found. 316.1408; anal. calcd. for $C_{20}H_{17}N_3O$: C, H, N.

4.1.3.4. Synthesis of 7-ethyl-3-(4-methoxy-phenyl)-2,7-dihydro-1H-pyrrolo[3,2-f]quinazolin-1-one (**22**)

The synthesis of compound **22** was carried out by the same procedure as for **18** by thermal cyclization of amidine derivative **17** (0.5006 g, 1.36 mmol). Yield 65%, brown solid; mp>300 °C; rf 0.70 (ethyl acetate/*n*-hexane 8:2); IR ν_{max} (KBr): 3217 (NH), 1680 (C═O) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.41 (*t*, 3H, J = 7.2 Hz, NCH_2CH_3), 3.83 (*s*, 3H, OCH_3), 4.34 (*q*, 2H, J = 7.2 Hz, NCH_2CH_3,), 7.08 (*d*, 2H, J = 9.0 Hz, 3′-H and 5′-H), 7.31 (dd, 1H, J = 3.05 and J = 0.6 Hz, 9-H), 7.51 (*d*, 1H, J = 8.8 Hz, 5-H), 7.62 (*d*, 1H, J = 3.1 Hz, 8-H), 8.02 (dd, 1H, J = 8.8 and J = 0.6 Hz, 6-H), 8.21 (d, 2H, J = 9.0 Hz, 2′-H and 6′-H), 12.31 (bs, 1H, *NHC*(O)); ^{13}C NMR (DMSO-d_6) δ 15.8, 40.6, 54.8, 102.9, 112.7, 115.1, 117.5, 120.6, 123.7, 127.5, 129.8, 130.8, 133.0, 133.1, 144.9, 149.6, 162.4; HRMS (ESI) $[M + H]^+$ calculated for $C_{19}H_{17}N_3O_2$ *m*/*z* $C_{19}H_{18}N_3O_2$ = 320,1354; found 320.1366; anal. calcd. for $C_{19}H_{17}N_3O_2$: C, H, N.

4.2. Biological assays

4.2.1. Preparation of platelet suspensions

Blood samples were collected from healthy volunteers who had taken no medications during the preceding 2 weeks, with their informed consent and in accordance with the Helsinki Declaration. Platelet isolation was performed by differential centrifugation, as previously described [39]. Briefly: blood was immediately mixed with citric-anticoagulant ACD (75 mM sodium citrate, 40 mM citric acid, 130 mM dextrose, pH 6.0), supplemented with 0.8 mU/mL apyrase and 20 mg/mL prostacyclin, and centrifuged for 20 min at 200 g. The supernatant platelet-rich plasma (PRP) was further centrifuged for 20 min at 750 g. Spun-down platelets were re-suspended, unless otherwise indicated, in basal buffer consisting of (mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, and 10 Tris/Hepes, (pH 7.4), at a concentration of $2 - 4 \times 10^8$ cells/mL. Where indicated, acetylsalicylic acid treatment was performed by addition (0.1 mM final concentration) of a freshly prepared solution in 0.1 M $NaHCO_3$ to platelet-rich plasma, followed by 20 min incubation at 36 °C.

4.2.2. Determination of platelet aggregation

Platelet aggregation was followed turbidometrically on an Elvi-Logos aggregometer as previously reported [40].

4.2.3. Analysis of cytosolic Ca^{2+} concentration

Cytosolic [Ca^{2+}] was determined by means of the fluorescent probe fura 2, as previously described [41]. Fluorescence changes were measured in a thermostated, magnetically stirred quartz cuvette, at dual excitation wavelengths of 340 and 385 nm and an emission wavelength of 505 nm. Calibration was performed with the calcium–ionophore ionomycin and calcium–chelator EGTA.

4.2.4. Detection of tyrosine phosphorylation of platelet proteins

Platelets suspended in the basal medium, at a count of 300×10^6 cells/mL, were incubated according to the experimental protocol. Aliquots of 30 μl (protein concentrations determined according to Bradford [42]) were withdrawn from the differently treated platelet suspensions and transferred into Eppendorf test tubes containing 15 μL of Laemmli solution supplemented with 2-mercaptoethanol (5%), anti-phosphatase sodium orthovanadate (1 mM), anti-protease EDTA (2 mM) and electrophoretic tracer pyronin. Samples were subjected to SDS/PAGE (10% gels) and separated proteins were immediately electrophoretically transferred to nitrocellulose membranes, treated with anti-phosphotyrosine PY20 (ICN Biotechnology), followed by secondary peroxidase-conJugate antibody and detected by the enhanced chemi-luminescence technique (ECL, Amersham Pharmacia Biotech, San Francisco, CA) [39]. Anti-βactin immunoblotting was performed in parallel to check the amounts of proteins in SDS/PAGE lanes.

4.2.5. Platelet damage

Possible damage caused by 3-PPyQZ on platelet membranes was analysed by monitoring the release of lactate dehydrogenase (LDH)] from platelets incubated with the compound [36]. LDH release was measured spectrophotometrically by recording β-NADH oxidation following pyruvate addition. Platelet suspensions (1 mL, 2×10^8 cells/mL) were incubated at 37 °C for different times, with various concentrations of tested compounds, and then

centrifuged for 3 min at 3000 g. The resulting LDH activity was determined in supernatants after addition of 5 mM β-NADH and 50 mM sodium pyruvate and recording of optical density decrease at 340 nm. Total cellular LDH activity was determined after platelet lysis with 0.1% Triton X-100.

Platelet viability was determined by the fluorimetric method, based on oxidation/reduction of the cellular indicator Alamar Blue [37]. In the latter case, platelet suspensions supplemented with 1 mM NaHCO3 and 0.2% bovine serum albumin were kept in a cellular incubator at 37 °C under 5% CO_2 flux.

4.2.6. Statistical analysis

Analysis of statistical significance of differences was performed with the non-parametric Mann–Whitney *U*-test.

Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.ejmech.2011.12.026.

References

[1] D.A. Horton, G.T. Bourne, M.L. Smythe, The combinatorial synthesis of bicyclic privileged structures or privileged substructures, Chem. Rev. 103 (2003) 893–930.
[2] S. Sinha, M. Srivastava, Biologically active quinazolones, Prog. Drug Res. 43 (1994) 143–238.
[3] I.K. Kacker, S.H. Zaheer, Potential analgesics. Part I. Synthesis of substituted 4-quinazolones, J. Ind. Chem. Soc. 28 (1951) 344–346.
[4] P.M. Dewick, in: P.M. Dewick (Ed.), Medicinal Natural Products: A Biosynthetic Approach, third ed. John Wiley & Sons, Ltd, Chichester, UK, 2009 pp. 395–397.
[5] A.M. Alafeefy, Synthesis and antimicrobial activity of some new quinazolin-4(3*H*)-one derivatives, Pharm. Biol. 46 (2008) 751–756.
[6] V. Alagarsamy, V.R. Solomovv, K. Dhanabal, Synthesis and pharmacological evaluation of some 3-phenyl-2-substituted-3*H*-quinazolin-4-one as analgesic, anti-inflammatory agents, Bioorg. Med. Chem. 15 (2007) 235–241.
[7] A. Kumar, C.S. RaJput, Synthesis and anti-inflammatory activity of newer quinazolin-4-one derivatives, Eur. J. Med. Chem. 44 (2009) 83–90.
[8] J. Kunes, M. Bazant, M. Pour, M. Waisser, M. Slosarek, J. Janota, Quinazoline derivatives with antitubercular activity, Il Farmaco 55 (2000) 725–729.
[9] J. Guan, Q. Zhang, M. O'Neil, N. Obaldia, A. Ager, L. Gerena, A.J. Lin, Antimalarial activities of new pyrrolo[3,2-*f*]quinazoline-1,3-diamine derivatives, Antimicrob. Agents Chemother. 49 (2005) 4928–4933.
[10] A. Kamal, E.V. Bharathi, M.R. Janaki, D. Dastagiri, R.J. Surendranadha, A. Viswanath, F. Sultana, S.N. Pushpavalli, M. Pal-Bhadra, H.K. Srivastava, S.G. Narahari, A. Juvekar, S. Sen, S. Zingde, Quinazoline-linked pyrrolo[2,1-*c*][1,4]benzodiazepine (BDP) conjugates: design, synthesis and biological evaluation as potential anticancer agents, Bioorg. Med. Chem. 8 (2010) 526–542.
[11] R.C. Morris, W.E. Hanford, R. Adams, Structure of vasicine. III. Position of the hydroxyl group, J. Am. Chem. Soc. 57 (1935) 951–954.
[12] R.K. Thappa, S.G. Agarwal, K.L. Dhar, V.K. Gupta, K.N. Goswami, Two pyrroloquinazolines from *Adhatoda vasica*, Phytochemistry 42 (1996) 1485–1488.
[13] H.D. Showalter, H.L. Sun, A.D. Sercel, R.T. Winters, W.A. Denny, B.D. Palmer, Concise syntheses of the novel 1*H*-pyrrolo[3,2-*g*]quinazolines ring system and its [2,3-*f*] angular isomer, J. Org. Chem. 61 (1996) 1155–1158.
[14] B.D. Palmer, S. Trump-Kallmeyer, D.W. Fry, J.M. Nelson, H.D.H. Showalter, W.A. Denny, Protein kinase inhibitors. 11. Soluble analogues of pyrrolo- and pyrazoloquinazolines as epidermal growth factor receptor inhibitors: synthesis, biological evaluation and modelling of the mode of binding, J. Med. Chem. 40 (1997) 1519–1529.
[15] M.G. Ferlin, R. Bortolozzi, P. Brun, I. Castagliuolo, E. Hamel, G. Basso, G. Viola, Synthesis and in vitro evaluation of 3*H*-pyrrolo[3,2-*f*]quinolin-9-one derivatives that show potent and selective anti-leukemic activity, Chem. Med. Chem. 5 (2010) 1373–1385.
[16] V. Gasparotto, I. Castagliuolo, M.G. Ferlin, 3-Substituted 7-phenyl-pyrroloquinolinones show potent cytotoxic activity in human cancer cell lines, J. Med. Chem. 50 (22) (2007) 5509–5513.
[17] M. Verstraete, P. Zoldhely, Novel antithrombotic drugs in development, Drugs 49 (1995) 856–884.
[18] H.S. Ahn, L. Arik, G. Boykow, D.A. Burnett, M.A. Caplen, M. Czarniecki, M.S. Domalski, C. Foster, M. Manna, A.W. Stamford, Y. Wu, Structure–activity relationship of pyrroloquinazolines as thrombin receptor antagonist, Bioorg. Med. Chem. Lett. 9 (1999) 2073–2078.
[19] A. Dixit, S.K. Kashaw, S. Gaur, A.K. Saxena, Development of CoMFA, advance CoMFA and CoMSIA models in pyrroloquinazolines as thrombin receptor antagonist, Bioorg. Med. Chem. 12 (2004) 3591–3598.
[20] M.G. Ferlin, M.T. Conconi, L. Urbani, B. Oselladore, D. Guidolin, R. Di Liddo, P. Parnigotto, Synthesis, *in vitro* and *in vivo* preliminary evaluation of anti-angiogenic properties of some pyrroloazaflavones, Bioorg. Med. Chem. 19 (2011) 448–457.
[21] L.-J. Huang, M.-C. Hsieh, C.-M. Teng, K.-H. Lee, S.-C. Kuo, Synthesis and anti-platelet activity of phenyl quinolones, Bioorg. Med. Chem. 6 (1998) 1657–1662.
[22] M.L. Jones, L.F. Kuyper, V.L. Styles, J.M. Caddel, Lewis acid assisted cyclization of arylcianoguanidines to 2,4-diaminoquinazolines, Heterocyclic Chem. 31 (1994) 1681–1683.
[23] W.D. Dean, E.P. Papadopoulos, N-ethoxycarbonylamidines as starting material of intermediates in the synthesis of heterocyclic compounds, J. Heterocyclic Chem. 19 (1982) 171–176.
[24] E.P. Papadopoulos, Friedel-Crafts thioacylation with ethoxycarbonyl isothiocyanate. A one-step synthesis of aromatic thioamides, J. Org. Chem. 41 (1976) 962–965.
[25] M.P. Cava, M.I. Levinson, Thionation reactions of Lawesson's reagent, Tetrahedron 41 (1985) 5061–5087.
[26] W. Siess, Molecular mechanisms of platelet activation, Physiol. Rev. 69 (1989) 58–178.
[27] G.R. Sambrano, E.J. Weiss, Y.W. Zheng, W. Huang, S.R. Coughlin, Role of thrombin signalling in platelets in haemostasis and thrombosis, Nature 413 (2001) 74–78.
[28] D.E. Roberts, A. McNicol, R. Bose, Mechanism of collagen activation in human platelets, J. Biol. Chem. 279 (2004) 19421–19430.
[29] F. Zoccarato, M. Ruzzene, L. Cavallini, M.G. Doni, M.A. Francesconi, R. Deana, A. Alexandre, Platelet responses promoted by the activation of protein kinase C or the increase of cytosolic Ca2+ are potentiated by adrenaline. Effects of cAMP and staurosporine, Biochim. Biophys. Acta 1092 (1991) 72–78.
[30] T.J. Rink, S.O. Sage, Calcium signalling in human platelets, Annu. Rev. Physiol. 52 (1990) 129–136.
[31] S.O. Sage, R. Reast, T.J. Rink, ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets. Evidence for Ca^{2+} entry regulated by the intracellualar Ca^{2+} stores, Biochem. J. 265 (1990) 675–680.
[32] A. Golden, J.S. Brugge, Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets, Proc. Natl. Acad. Sci. USA 86 (1989) 901–905.
[33] S. Nakamura, H. Yamamura, Thrombin and collagen induce rapid phosphorylation of a common set of cellular proteins on tyrosine in human platelets, J. Biol. Chem. 264 (1989) 7089–7091.
[34] E.A. Clark, S.J. Shattil, J.S. Brugge, Regulation of protein tyrosine kinase in platelets, Trends Biochem. Sci. 19 (1994) 464–469.
[35] G.W. Rewcastle, B.D. Palmer, A.J. Bridges, H.D. Showalter, L. Sun, J. Nelson, A. McMichael, A.J. Kraker, D.W. Fry, W.A. Denny, Tyrosine kinase inhibitors. 9. Synthesis and evaluation of fused tricyclic quinazoline analogues as ATP site inhibitors of the tyrosine kinase activity of the epidermal growth factor receptor, J. Med. Chem. 39 (1996) 918–928.
[36] Y.-R. Jin, C.-K. Ryu, C.-K. Moon, M.-R. Cho, Y.-P. Yun, Inhibitory effects of J78, a newly synthesized 1,4-naphtoquinone derivative, on experimental thrombosis and platelet aggregation, Pharmacology 70 (2004) 195–200.
[37] S.A. Back, R. Khan, X. Gan, P.A. Rosenberg, J.J. Volpe, A new Alamar blue viability assay to rapidly quantify oligodendrocyte death, J. Neurosci. Meth. 91 (1999) 47–54.
[38] P. Zania, S. Kritikou, C.S. Flordellis, M.E. Maragoudakis, N.E. Tsopanoglou, Blockade of angiogenesis by small molecule antagonists to protease-activated receptor-1: association with endothelial cell growth suppression and induction of apoptosis, J. Pharmacol. Exp. Ther. 318 (2006) 246–254.
[39] A. Zarpellon, A. Donella-Deana, A. Folda, L. Turetta, M. Pavanetto, R. Deana, Serotonin (5-HT) transport in human platelets is modulated by Src-catalysed Tyr-phosphorylation of the plasma membrane transporter SERT, Cell. Physiol. Biochem. 21 (2008) 87–94.
[40] M.G. Doni, R. Deana, S. Bertoncello, F. Zoccarato, A. Alexandre, Forskolin and prostacyclin inhibit fluoride induced platelet activation and protein kinase C dependent responses, Biochem. Biophys. Res. Commun. 156 (1988) 1316–1323.
[41] L. Turetta, E. Bazzan, K. Bertagno, E. Musacchio, R. Deana, Role of Ca(2+) and protein kinase C in the serotonin (5-HT) transport in human platelets, Cell Calcium 31 (2002) 235–244.
[42] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.