European Journal of Medicinal Chemistry 57 (2012) 391–397

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

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

Short communication

Synthesis of novel pyrrolyl-indomethacin derivatives

Judith Serra Moreno [a], Dimitrios Agas [a,c], Maria Giovanna Sabbieti [c], Matteo Di Magno [a], Antonella Migliorini [a], M. Antonietta Loreto [a,b,*]

[a] Chemistry Department, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy
[b] Istituto di Chimica Biomolecolare, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy
[c] School of Biosciences and Biotechnology, University of Camerino, Via Gentile III da Varano, 62032 Camerino, Italy

ARTICLE INFO

Article history:
Received 19 December 2011
Received in revised form
3 September 2012
Accepted 5 September 2012
Available online 12 September 2012

Keywords:
Indomethacin
NSAID
Ester
Pyrrole
Osteoblasts
Cytotoxicity

ABSTRACT

In the present work, we report the synthesis of the novel esters of indomethacin (IDMC) and an ester of reduced IDMC. For this purpose, IDMC is covalently bound by using a spacer chain to the pyrrole (Py) in the 3-position. The innovative pyrrole-indomethacin (3-Py-IDMC) derivates show no cytotoxic effects in primary calvarial osteoblasts. The designed IDMC derivates have been studied because they could be injected locally as a component of polymeric micro-particles. In fact, the new 3-Py-IDMC derivatives will assure their further polymerization since the 2- and 5-monomer positions are free.

1. Introduction

Indomethacin (IDMC) is a non-steroidal anti-inflammatory drug (NSAID) commonly used for the treatment of pain and inflammation by inhibiting the cyclooxygenases (COXs) activities (Fig. 1) [1].

The major drawback of IDMC, if orally ingested, is an undesirable gastric effect. The intra-articular (IA) administration of NSAIDs is a valid alternative to minimize the systemic bioavailability and associated side-effects of oral intake. However, the IA therapy for osteoarthritis still needs micro-/nano-carrier mediated drug delivery systems to decrease the drug loading, minimizing the negative side effects, and to increase the drug bioavailability over time. In fact, the latter aspect is the key point for a successful drug delivery carrier. The current NSAIDs delivery vehicles still show a rapid clearance of therapeutic substances from the synovial space [2]. In the literature different methods to immobilize NSAIDs into polymers are proposed such as encapsulation technique [3–5]. In this perspective, rather to encapsulate the drug, we plan to bind IDMC derivatives namely, esters of IDMC and an ester of reduced IDMC, to a monomer for further polymerization as nano-/micro-particles. Therefore, the designed polymer is expected to decrease the drug clearance. The polymeric sample could be locally injected to the painful zone, overcoming the oral IDMC administration problems, and could maintain the bioavailability. The chosen monomer is pyrrole that leads to polypyrrole (PPy) that is a well known polymer for its electroconducting properties and biomedical applications [6]. For instance, PPy can be used as a reservoir for drug delivery following a redox reaction [7,8].

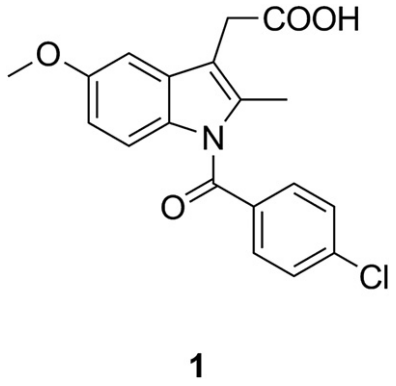
In this work, we report the covalent binding of IDMC or reduced IDMC to 3-position of pyrrole (3-Py-IDMC also referred as IDMC derivates) through a hydrolyzable spacer chain by following esters (**5**) and (**8**) and ester of reduced IDMC (**13**) strategies, respectively. The modified 3-Py-IDMC monomers were further studied by performing cytotoxic tests in vitro. The designed monomers will be copolymerized with pyrrole monomer (3-Py-IDMC/Py) in order to obtain micro-particles that will be used for IDMC or reduced IDMC delivery *in situ*. It is expected that the covalent immobilization of IDMC or reduced IDMC to the 3-Py could assure their controlled release from the copolymer micro-particles in respect to the direct IDMC injection.

2. Results and discussion

The indomethacin ester **5** is carried out by a 3-step synthesis (Scheme 1).

* Corresponding author. Chemistry Department, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy. Tel.: +39 06 4991 3675; fax: +39 06 490631.
E-mail address: mariaantonietta.loreto@uniroma1.it (M.A. Loreto).

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2012.09.008



1

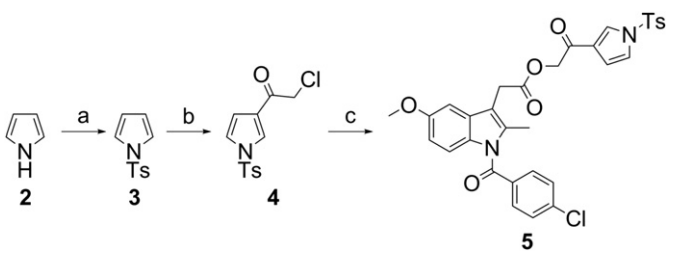
Fig. 1. Chemical structure of indomethacin.

The first step was the *N*-tosylation of the pyrrole that was easily obtained by tosyl chloride in the presence of sodium hydroxide [9]. Afterward the chloroacetyl chloride reacted with the *N*-protected pyrrole **3** following the condition for the simple acetylation of the *N*-tosylpyrrole, using $AlCl_3$ in dry dichloromethane [10]. The 3-chloroacetyl *N*-tosylpyrrole **4** was obtained in 60% yield, the 2-acylated isomer was also present in 19% yield. The two products were separated by chromatography and recognized by ^1H NMR spectra [11]. The last step, that is the reaction of the 3-chloroacetyl pyrrole *N*-protected with IDMC, was performed successfully by using the *N*,*N*-dimethyl-4-aminopyridine (DMAP) as the coupling agent and the triethylamine as the base in agreement with reported procedure for the glicolamide ester of indomethacin [12]. Dichloromethane was used as the solvent. The product **5** was purified and obtained in 84% yield. The structure was confirmed by spectroscopic data. The ^1H NMR spectrum shows the methylene protons of $-COOCH_2CO-$ group at δ 5.0. The ^{13}C NMR spectrum confirms the expected number of C atoms and the ester bond at ~170 ppm.

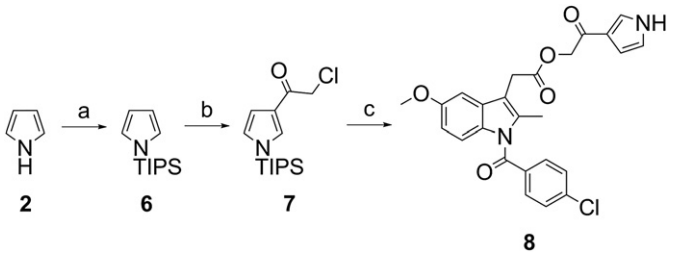
In addition, it was possible to obtain the IDMC ester **8** with the unprotected pyrrole starting from the *N*-triisopropylsilyl (*N*-TIPS) pyrrole **6** that was prepared according to reported procedure [13], as outlined in Scheme 2.

The reaction of **6** with chloroacetyl chloride lead to the new 3-chloroacetyl pyrrole **7** in the same conditions reported for Py acylation but by using different acyl chlorides [14]. The compound **7** was obtained as the main product in 43% yield and a small quantity (16%) of the 2-acylated N–H pyrrole (pyrrole *N*-unprotected) was also isolated. The last step was the coupling reaction carried out in the same conditions of those for the synthesis of compound **5**. These conditions facilitated the deprotection of the monomer providing the desired product **8**. The ^1H NMR and ^{13}C NMR analyses confirmed the absence of the TIPS group and the success of the last reaction even if with modest yield (40%).

The two obtained esters **5** and **8** are new esters of IDMC. Both oxoethylesters could allow the chemical or enzymatic hydrolysis of IDMC as reported for the IDMC 2-oxopropylester derivates at



Scheme 1. Synthesis of indomethacin ester **5**. Reagents and conditions: (a) TsCl, NaOH, DCE, 0 °C; (b) $ClCH_2COCl$, $AlCl_3$, CH_2Cl_2, r.t.; (c) indomethacin **1**, DMAP, Et_3N, CH_2Cl_2, r.t.



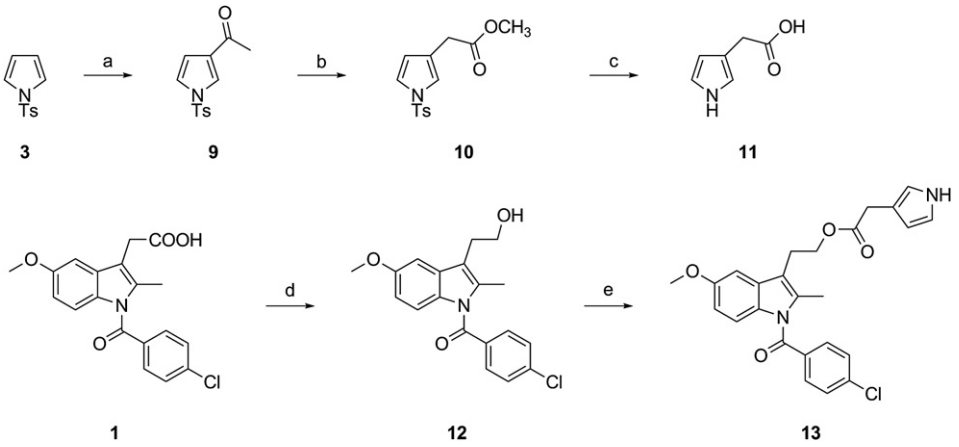
Scheme 2. Synthesis of indomethacin ester **8**. Reagents and conditions: (a) TIPSCl, *n*-BuLi, THF, −78 °C; (b) $ClCH_2COCl$, $AlCl_3$, CH_2Cl_2, 0 °C; (c) indomethacin **1**, DMAP, Et_3N, CH_2Cl_2, r.t.

selected conditions proposing them as potential prodrugs [15]. The products **5** and **8** leave free the positions 2- and 5- of the Py. It is well known that the substitution of a functional group on the *N*- or the 3-position of the pyrrole allows its polymerization if the substituent is not too bulky [16]. In fact, the compounds **5** and **8** can assure their further polymerization because they use a spacer chain that holds indomethacin moiety far from polymerization center.

A different spacer was used for connecting the reduced indomethacin (alcohol **12**) to 3-position of Py to give the ester derivative **13**. The synthesis pathway followed is depicted in Scheme 3.

The first steps concern the synthesis of the 3-acetic acid pyrrole **11** (key intermediate) that involves the acetylation of the *N*-tosylpyrrole **3** and the conversion of acetylated **9** into the 3-acetic acid methyl ester **10** by using the thallium-based oxidative transposition. The oxidative transposition was achieved by the use of thallium trinitrate ($Tl(NO_3)_3$), trimethyl orthoformate and montmorillonite K10 [17]. The following hydrolysis leads to the targeted key intermediate **11**. On the other hand, the IDMC was reduced to the corresponding alcohol **12** by using the borane–methyl sulfide complex ($BH_3 \cdot SMe_2$) in THF according to the Wey's method [18]. The reduced indomethacin was produced with high yield (91%). It was condensed with the 3-acetic acid pyrrole **11** employing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and *N*,*N*-dimethyl-4-aminopyridine (DMAP) as showed in the Kalgutkar's procedure for other different reduced IDMC esters [19]. The synthesized ester **13** was isolated by chromatography with a yield of 62% and its structure was confirmed by spectroscopic techniques. The ^1H NMR spectrum showed the signals of the reduced IDMC ester and the 3-pyrrole acetate. The obtained structure **13** could allow its polymerization as well as **5** and **8** compounds.

The innovative monomers were tested in order to study their biocompatibility using primary calvarial osteoblasts as a cell model. Since we plan to copolymerize them, it is useful to characterize their cytotoxicity whenever IDMC, reduced IDMC or un-reacted 3-Py-IDMC monomer could be released from the copolymer. In fact, it is expected that the delivery of these compounds is due to the hydrolysis of the ester bond. The hydrolysis of the ester bond is a well known strategy to release drugs in vitro or in vivo [20–22]. MTS assay, a colorimetric method that determines the number of viable cells, showed that treatment with the compounds **5**, **8**, **13** and IDMC did not affect osteoblast viability (Fig. 2A). In particular, with Hoechst staining and with BrdU assay, performed to specifically evaluate the cell proliferation, no statistically significant differences were found between compounds-stimulated and unstimulated cultures (Fig. 2B_1, B_2). Moreover, the synthesis of the anti-apoptotic protein Bcl2 and the pro-apoptotic protein Bax was evaluated by western blotting, since a small change in the ratio of above proteins could reflect a cell perturbation. The obtained results demonstrated that Bcl2/Bax ratio was not affected by the tested compounds or by IDMC (Fig. 2C).



Scheme 3. Synthesis of reduced IDMC ester **13**. Reagents and conditions: (a) Ac_2O, $AlCl_3$, CH_2Cl_2, r.t.; (b) $Tl(NO_3)_3 \cdot 3H_2O$, montmorillonite K10, $CH(OCH_3)_3$, CH_3OH, r.t.; (c) 1) NaOH, CH_3OH, reflux; 2) HCl, r.t.; (d) $BH_3 \cdot S(CH_3)_2$, THF, r.t.; (e) 2-(1*H*-pyrrol-3-yl)acetic acid **11**, EDCI, DMAP, CH_2Cl_2, r.t.

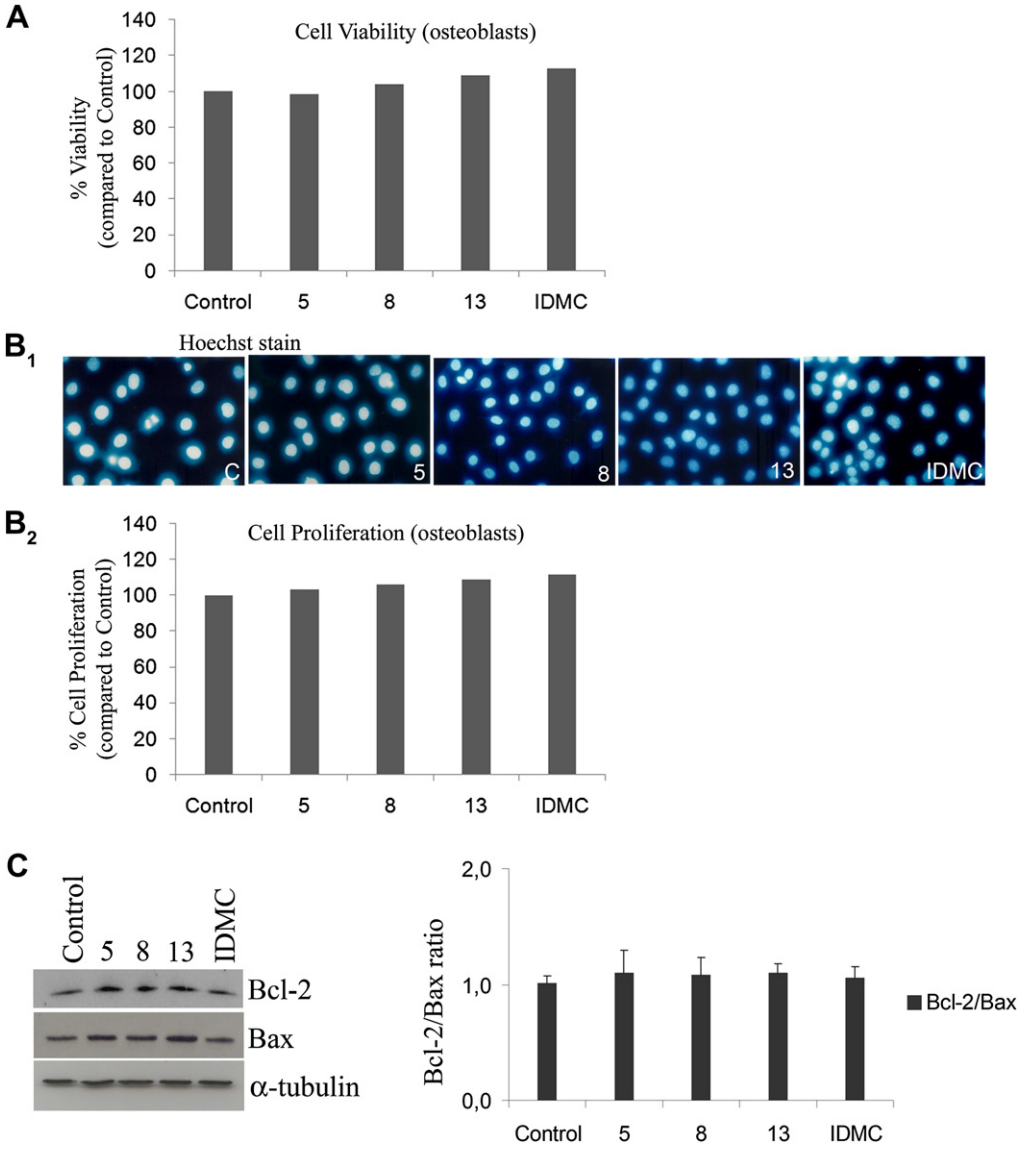


Fig. 2. MTS assay (A). COBs were incubated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M), for 24 h. Graphics represent results of three independent experiments. Hoechst stain (B_1); BrdU assay (B_2). Cells were treated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M), for 24 h. Graphic represents results of three independent experiments. Bcl2/Bax ratio (C). Cells were treated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24 h. Filters were probed with the rabbit anti-Bcl2 or with the rabbit anti-Bax; then, membranes were stripped and re-probed with mouse anti-α-tubulin to show equal amount of loading. Graphic represents results of three independent experiments.

It is well known that the cytoskeleton plays a crucial role not only in cell shaping, but also in many cellular functions. In osteoblastic cells cytoskeletal modifications are helpful tools for monitoring cell healthiness. Indeed, integrity of the osteoblast structure, is important for their differentiation as well as for the activation of osteoclast functions through a mechanism involving cell-to-cell and/or cell-to-matrix contacts [23]. Therefore, the distribution of F-actin was investigated by using TRITC-phalloidin labeling. The results evidenced a defined organization of actin cytoskeleton in osteoblasts treated with compounds **5**, **8**, **13** and IDMC, overlapping to that found in untreated cells (Fig. 3A). Moreover, toluidine blue and hematoxylin/eosin cytochemical staining, performed to confirm the above results, showed that the fusiform or polygon shape of the cells observed in untreated osteoblasts was preserved after administration of the tested compounds (Fig. 3B, C).

Hence, the above multiple experimental approaches strongly evinced that these novel 3-Py-IDMC monomers are biocompatible and could be useful for further copolymerization to obtain microparticles for IDMC or reduced IDMC delivery. It will be attended a drug release by the linker hydrolysis (ester bond). These IDMC derivates could represent a novel bases for the preparation of Py-IDMC-based polymeric drugs for local application with less side effects than the existing oral administrated NSAIDs.

3. Conclusions

In summary, in this work we have reported the synthesis of the innovative IDMC-based compounds namely, IDMC esters and reduced IDMC ester. From our knowledge, this is the first time that IDMC is covalently bound by using a spacer chain to the Py in the 3-position (3-Py-IDMC). In addition, the innovative pyrrole-indomethacin monomers showed no cytotoxic effects by using primary calvarial osteoblasts. It could be attended, therefore, no cytotoxic effects also for the respective polymer. The new 3-Py-IDMC derivatives assure their further polymerization since the 2- and 5-monomer position are free. The obtained polymers will be new interesting materials potentially applicable in pharmacological field.

4. Experimental section

4.1. General experimental methods

Solvents and common reagents were purchased from a commercial source and used without further purification. All reactions were monitored by thin layer chromatography (TLC) carried out on Merck F-254 silica glass plates and visualized with UV light. ^{1}H NMR was recorded in $CDCl_3$ on Varian Gemini 300 (300 MHz). Chemical shifts are expressed in parts per million (δ scale) and are referenced to the residual protons of the NMR solvent ($CHCl_3$: δ 7.26); (s) = singlet; (d) = doublet; (t) = triplet; (q) = quartet; (dd) = double doublet; (ddd) = double double doublet; (dt) = double triplet; (dq) = double quartet; (bs) broad singlet; (m) = multiplet. Coupling constants (*J*) were expressed in Hz. ^{13}C NMR was recorded in $CDCl_3$ on Varian Gemini 300 (75 MHz). Chemical shifts are expressed in parts per million (δ scale) and are referenced to the residual carbons of the NMR solvent ($CHCl_3$: δ = 77.0). Infrared spectra (IR) were obtained using a PERKIN–ELMER 1600 (FT-IR); data are presented as the frequency of absorption (cm^{-1}). HRMS spectra were recorded with Micromass Q-TOF *micro* Mass Spectrometer (Waters). GC–MS spectra were recorded with SHIMAZU GC-2010 equipped with a capillary column SLB-5ms (30 m × 0.25 mm × 0.25 μm) coupled with a detector SHIMAZU QP 2010S.

4.1.1. 3-(Chloroacetyl)-1-tosyl-1H-pyrrole (**4**)

Chloroacetyl chloride (1.32 g, 11.8 mmol) was added slowly to a suspension of anhydrous $AlCl_3$ (2.23 g, 16.7 mmol) in 20 mL of dry dichloromethane at 25 °C. The reaction mixture was stirred at r.t. for 10 min, a solution of *N*-tosylpyrrole **3** (0.99 g, 4.5 mmol) in 2.5 mL of dry dichloromethane was added slowly and the mixture was stirred for 2 h at r.t and then poured into an ice–water mixture. The organic phase was separated and combined with dichloromethane extracts (4 × 10 mL) of the aqueous phase. The combined organic layer was washed with NaOH solution (1 N), dried (Na_2SO_4 anhydrous) and evaporated in vacuo. The residue was chromatographed on silica gel (dichloromethane/hexane, 7/3) to afford the

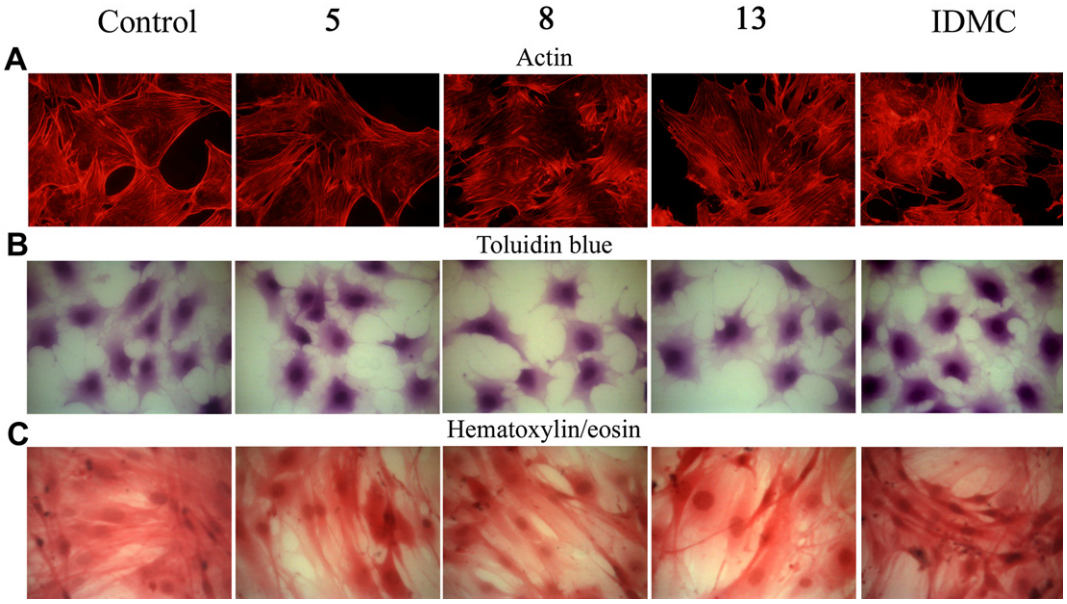


Fig. 3. TRITC-phalloidin labeling for F-actin (A). Cells were incubated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24 h. Stress fibers are seen running parallel to the long axis of the cells and in the cytoplasmic projections both in treated and in untreated cells. Toluidine blue (B); hematoxylin/eosin (C). Cells were incubated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24 h. Treated cells are morphologically comparable to control cultures.

pure 3-chloroacetyl *N*-tosylpyrrole **4** as a white solid in 60% yield, the 2-acylated isomer was also present in 19% yield. The compound **4** was recognized by ^{1}H NMR spectra [11].

4.1.2. 2-Oxo-2-(1-tosyl-1H-pyrrol-3-yl)ethyl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate (**5**)

3-(Chloroacetyl)-1-tosyl-1*H*-pyrrole **4** (560 mg, 1.9 mmol) was added to a solution of indomethacin **1** (673 mg, 1.9 mmol), DMAP (46 mg, 0.4 mmol) in Et_3N (12 mL) and dichloromethane (12 mL). The reaction mixture was stirred at r.t. under argon atmosphere. After 24 h evaporation of the solvent in vacuo followed by flash chromatography on silica gel (hexane/ethyl acetate = 1/1) afforded the pure product **5** as a yellow solid (yield 991 mg, 1.6 mmol, 84%); R_f = 0.50 (hexane/ethyl acetate, 6/4). ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 2.34 (s, 3H, CH_3CN); 2.38 (s, 3H, CH_3CCH_{arom}); 3.79 (s, 2H, CCH_2CO); 3.81 (s, 3H, OCH_3); 5.04 (s, 2H, OCH_2CO); 6.61 (dd, J = 3.3 Hz, J = 1.6 Hz, 1H, CH_{py}); 6.65 (dd, J = 9.0 Hz, J = 2.5 Hz, 1H, CH_{arom}); 6.88 (d, J = 9.0 Hz, 1H, CH_{arom}); 6.99 (d, J = 2.5 Hz, 1H, CH_{arom}); 7.11 (dd, J = 3.3 Hz, J = 2.1 Hz, 1H, CH_{py}); 7.27–7.31 (m, 2H, CH_{arom}); 7.40–7.44 (m, 2H, CH_{arom}); 7.61–7.65 (m, 2H, CH_{arom}); 7.73–7.77 (m, 3H, CH_{py} + CH_{arom}) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 13.3; 21.6; 29.7; 55.6; 66.4; 101.1; 111.8; 112.0; 114.8; 121.7; 124.0; 125.1; 127.2; 129.0; 130.3; 130.5; 130.7; 131.1; 133.8; 134.7; 135.9; 139.1; 146.1; 156.0; 168.2; 170.2; 187.1 ppm. HRMS: calcd. for $[C_{32}H_{27}ClN_2O_7S + Na]^+$ 641.1120; found 641.1124. IR ($CHCl_3$): $\tilde{\nu}$ = 1746, 1690 cm^{-1}. M.p.: 134–136 °C.

4.1.3. N-(Triisopopylsilyl)pyrrole (**6**)

n-Butyllithium in hexane (2.2 mL of a 2.5 M solution, 5.5 mmol) was added dropwise to argon-dried pyrrole (compound **2**) (335 mg, 5.0 mmol) in 10 mL of anhydrous THF at −78 °C. Triisopropylsilyl chloride (965 mg, 5.0 mmol) was added after 10 min and the reaction warmed to room temperature. The solvent was then removed, water was added, and the resulting residue was extracted with diethyl ether. The organic phase was then dried over anhydrous sodium sulfate and concentrated by rotatory evaporation. *N*-(Triisopropylsilyl)pyrrole (**6**) was isolated as a colorless oil (yield 972 mg, 4.4 mmol, 87%); R_f: 0.48 (petrol ether 40–70 °C). The product was recognized by ^{1}H NMR spectra [13].

4.1.4. 3-(Chloroacetyl)-1-(triisopropylsilyl)-1H-pyrrole (**7**)

Chloroacetyl chloride (168 mg, 1.50 mmol) was added slowly to a stirred slurry of aluminum chloride (125 mg, 1.1 mmol) in anhydrous dichloromethane (2.0 mL at 0 °C). After 0.25 h, a solution of **6** (223 mg, 1.0 mmol) in dichloromethane (0.5 mL) was added. The mixture was stirred for 0.5 h at 0 °C and 0.5 h at r.t. and then poured into an ice–water mixture. The organic phase was separated and combined with a dichloromethane extract (4 × 5 mL) of the aqueous phase. The organic phase was dried (Na_2SO_4 anhydrous) and evaporated in vacuo. The residual material was subjected to flash chromatography on silica gel (hexane/ethyl acetate = 9/1). Compound **7** was obtained as a colorless oil (yield 128 mg, 0.43 mmol, 43%); R_f = 0.59 (hexane/ethyl acetate, 7/3). ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 1.10 (d, J = 7.5 Hz, 18H, $CH(CH_3)_2$); 1.47 (heptuplate, J = 7.5 Hz, 3H, $CH(CH_3)_2$); 4.47 (s, 2H, $ClCH_2CO$); 6.71 (dd, J = 2.9 Hz, J = 1.5 Hz, 1H, *CH*CHN); 6.75 (dd, J = 2.9 Hz, J = 2.0 Hz, 1H, CHC*H*N); 7.49 (dd, J = 2.0 Hz, J = 1.5 Hz, 1H, CCHN) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 11.5; 17.6; 46.3; 110.8; 125.8; 130.4; 133.0; 186.7 ppm. GC–MS: m/z 299 [M$^+$], 250 (100). IR ($CHCl_3$): $\tilde{\nu}$ = 1678 cm^{-1}.

4.1.5. 2-Oxo-2-(1H-pyrrol-3-yl)ethyl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetate (**8**)

Compound **8** was prepared from indomethacin **1** (139 mg, 0.4 mmol), 3-(chloroacetyl)-1-(triisopropylsilyl)-1*H*-pyrrole **7** (118 mg, 0.4 mmol), DMAP (9.5 mg, 0.1 mmol), Et_3N (1 mL) and dichloromethane (1 mL) according to the procedure described for **5**. After solvent evaporation, the crude product was purified by column chromatography on silica gel (hexane/ethyl acetate 6/4). The obtained product was already deprotected (no TIPS was present in the molecule). Compound **8** was obtained as a yellow solid (yield 74 mg, 0.16 mmol, 40%); R_f = 0.50 (hexane/ethyl acetate, 1/1). ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 2.37 (s, 3H, CH_3CN); 3.84 (s, 2H, CCH_2CO); 3.85 (s, 3H, OCH_3); 5.10 (s, 2H, OCH_2CO); 6.61 (td, J = 3.0 Hz, J = 1.6 Hz, 1H, CH_{py}); 6.68 (dd, J = 9.0 Hz, J = 2.5 Hz, 1H, CH_{arom}); 6.74 (dt, J = 3.0 Hz, J = 1.6 Hz, 1H, CH_{py}); 6.92 (d, J = 9.0 Hz, 1H, CH_{arom}); 7.04 (d, J = 2.5 Hz, 1H, CH_{arom}); 7.32 (dt, J = 3.0 Hz, J = 1.6 Hz, 1H, CH_{py}); 7.44–7.48 (m, 2H, CH_{arom}); 7.63–7.68 (m, 2H, CH_{arom}); 8.77 (bs, 1H, NH) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 13.4; 30.0; 55.8; 66.9; 101.4; 108.5; 112.0; 112.4; 114.9; 119.6; 122.3; 123.1; 129.1; 130.7; 130.9; 131.2; 133.9; 136.0; 139.3; 156.2; 168.4; 170.3; 187.7 ppm. HRMS: calcd. for $[C_{25}H_{21}ClN_2O_5 + Na]^+$ 487.1031; found 487.1036. IR ($CHCl_3$): $\tilde{\nu}$ = 3466, 1742, 1680 cm^{-1}. M.p.: 125–127 °C.

4.1.6. N-Tosyl-3-acetyl pyrrole (**9**)

Acetic anhydride (0.44 mL, 4.7 mmol) was added dropwise to a solution of aluminum chloride (896 mg, 6.7 mmol) in 5.22 mL of dichloromethane. After 10 min of stirring at room temperature, *N*-tosylpyrrole **3** (400 mg, 1.81 mmol) in 0.9 mL dichloromethane was added. The mixture was stirred for 2 h and poured into water and ice. The aqueous phase was extracted with CH_2Cl_2, washed with an aqueous solution of 1 N NaOH and dried over Na_2SO_4. The product was washed with methanol and after evaporation gave 99% of *N*-tosyl-3-acetyl pyrrole **9** as pure solid product (470 mg, 1.78 mg). R_f = 0.5 (hexane/ethyl acetate, 6/4). The product was recognized by ^{1}H NMR spectra [24].

4.1.7. N-Tosyl-3-methylacetate pyrrole (**10**)

Thallium trinitrate $Tl(NO_3)_3 \cdot 3H_2O$ (1.6 g, 3.6 mmol), trimethyl orthoformiate (4 mL) and montmorillonite K10 (3.2 g) were stirred in 3.3 mL methanol at room temperature for 45 min. The solvent was removed to obtain a powder completely dried. *N*-Tosyl-3-acetyl pyrrole **9** (470 mg, 1.79 mmol) and the catalyst were stirred for 12 h in 29 mL of methanol. The white precipitate of tallium salt was filtered and washed with a solution of dichloromethane:methanol (99:1). The solution was evaporated and filtered. The operation was repeated until the majority of the precipitate disappeared. 10 mL of diethyl ether was added and the solution was washed with water and a saturated NaCl solution, dried over Na_2SO_4 and evaporated. The yellow oil obtained was purified by silica gel column chromatography with heptane/ethyl acetate 9:1 to 7.5:2.5 as eluent to give *N*-tosyl-3-methylacetate pyrrole **10** (yield 260.8 mg, 50%). The product was recognized by ^{1}H NMR spectra [17].

4.1.8. 3-Acetic acid pyrrole (**11**)

2.87 mL NaOH (5 N) in 2.30 mL methanol was added to *N*-tosyl-3-methylacetatel pyrrole **10** (260.8 mg, 0.89 mmol) and refluxed for 2 h. The methanol was removed and the residue was washed with diethyl ether and acidified by using a gradient of aqueous HCl in the range of 5 N–0.5 N to pH 3, and finally extracted four times with diethyl ether. The solution was dried over Na_2SO_4 anhydrous. After removal of the solvent, 3-acetic acid pyrrole **11** was obtained as a white solid (yield 105 mg, 0.84 mmol, 94%). The product was recognized by ^{1}H NMR spectra [16].

4.1.9. Reduced indomethacin (**12**)

A solution of $BH_3 \cdot SMe_2$ in 2 M THF (0.8 mL, 1.6 mmol) was added slowly to an ice-cold solution of indomethacin (500 mg,

1.4 mmol) in THF (7.5 mL). The reaction was warmed up slowly from the ice-bath and stirred at room temperature for 22 h. Excess BH_3 was destroyed by the slow addition of MeOH (0.1 mL). The solvent was removed under reduced pressure. The crude material was dissolved in CH_2Cl_2 (10 mL), washed with saturated $NaHCO_3$ solution, water, 3 N HCl, water, and saturated NaCl solution, dried over Na_2SO_4 anhydrous. The yellowish solid **12** was collected and dried under vacuum (yield 439 mg, 1.3 mmol, 91%). The product was recognized by ^{1}H NMR spectra [18].

*4.1.10. 2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)ethyl 2-(1H-pyrrol-3-yl)acetate (**13**)*

EDCI (183 mg, 1.0 mmol), DMAP (6 mg, 0.05 mmol), and **12** (168 mg, 0.3 mmol) were added to a solution of 2-(1*H*-pyrrol-3-yl) acetic acid **11** (54 mg, 0.4 mmol) in dichloromethane (1.2 mL) and stirred overnight at r.t.. Upon dilution with water, the aqueous solution was extracted with dichloromethane (4 × 4 mL). The combined organic layer was washed with water (4 mL), dried (Na_2SO_4), filtered, and the solvent concentrated in vacuo. The residue was chromatographed on silica gel (hexane/ethyl acetate = 7/3) to afford the esters **13** as a yellow oil (yield 120 mg, 0.3 mmol, 62%); R_f = 0.38 (hexane/ethyl acetate, 6/4). ^{1}H NMR ($CDCl_3$, 300 MHz, 25 °C): δ = 2.32 (s, 3H, CH_3CN); 3.00 (t, J = 7.2 Hz, 2H, CCH_2CH_2O); 3.51 (s, 2H, CCH_2CO); 3.84 (s, 3H, OCH_3); 4.29 (t, J = 7.2 Hz, 2H, CCH_2CH_2O); 6.13–6.15 (m, 1H, CH_{arom}); 6.65–6.68 (m, 2H, CH_{arom}); 6.71–6.73 (m, 1H, CH_{arom}); 6.89 (d, J = 8.6 Hz, 1H, CH_{arom}); 6.97 (d, J = 2.5 Hz, 1H, CH_{arom}); 7.45–7.48 (m, 2H, CH_{arom}); 7.62–7.65 (m, 2H, CH_{arom}); 8.11 (bs, 1H, NH). ^{13}C NMR ($CDCl_3$, 75 MHz, 25 °C): δ = 13.1; 23.7; 33.0; 55.7; 63.5; 101.3; 109.1; 111.4; 114.9; 115.3; 115.4; 116.5; 118.0; 129.0; 130.9; 131.0; 131.0; 134.1; 135.2; 139.0; 156.0; 168.2; 172.4. HRMS: calcd. for $[C_{25}H_{23}ClN_2O_4 + Na]^+$ 473.1239; found 473.1245. IR ($CHCl_3$): $\tilde{\nu}$ = 3481, 1730, 1674 cm^{-1}. M.p.: 90–92 °C.

4.2. Experimental animals

Harlan Sprague–Dawley ICR (CD-1) male mice (Harlan, Italy) were used. Mice were sacrificed by CO_2 narcosis and cervical dislocation in accordance with the recommendation of the Italian Ethical Committee and under the supervision of authorized investigators.

4.2.1. Primary calvarial osteoblasts (COBs)

COBs were obtained from newborn mice by sequential digestion with 0.1% collagenase (Roche Diagnostic, Milano, Italy) [25]. Cells were pooled and cultured to confluence in 100-mm dishes and grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen S.R.L. Milano, Italy) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen S.R.L. Milano, Italy), penicillin (100 U/ml), and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C [26].

4.2.2. Assessment of viable COBs (MTS assays)

The metabolic activity of viable COBs was determined by the MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay as previously described [27]. Briefly, COBs were plated at the density of 5000 cells/well in 96 culture dishes (Costar Corp., Celbio, Italy) and grown DMEM, supplemented with 10% heat inactivated FCS, penicillin and streptomycin to approximately 80% confluence. Then, cells were treated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24. Subsequently, cells were incubated with 20 μl/well of CellTiter 96 AQueous One Solution Reagent (Promega Italia srl, Milano, Italy) for 2 h in a humidified, 5% CO_2, atmosphere. The quantity of formazan product is directly proportional to the number of living cells in culture. The colored formazan was measured by reading the absorbance at 490 nm using a 96 well plate reader. The experiment was run in triplicate.

4.2.3. Hoechst staining

In order to stain cell nuclei, Hoechst staining was performed using a 1:800 dilution from 2 mmol/l Hoechst dye stock (Sigma–Aldrich, Milano, Italy) on COBs treated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24.

4.2.4. BrdU assay

BrdU assay was performed using "Cell Proliferation ELISA, BrdU (colorimetric)" (Roche Diagnostics). Cells were treated for 24 h with compounds **5**, **8** and **13** (10^{-6} M) and IDMC (10^{-6} M). Then cultures were labeled with BrdU following the manufacturer's instructions. Photometric detection was done with an ELISA reader at 370 nm wavelength. The experiment was run in triplicate.

4.2.5. Western blotting

COBs were plated in 6-well culture dishes (Costar Corp. Celbio, Italy) at the density of 15,000 cells/cm^2 and grown for 5–6 days in DMEM with 10% heat inactivated FCS, penicillin and streptomycin. Then, cells were treated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24. Proteins were extracted with Cell Lysis Buffer (EuroClone, Milano, Italy) and the concentration was determined by the BCA protein assay reagent (Pierce, Celbio, Italy). After SDS-polyacrylamide gel electrophoresis on 12% gels, proteins were transferred to PVDF membranes (Bio-Rad, Milano, Italy). The next steps were performed by ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Europe, GMBH). Briefly, membranes were blocked with Advance Blocking Agent in PBS-T (PBS containing 0.1% Tween 20) for 1 h at room temperature. Then, membranes were incubated with a rabbit anti-Bcl-2 antibody (Santa Cruz Biotechnology, Inc., Italy) or with a rabbit anti-Bax antibody (Santa Cruz Biotechnology, Inc., Italy) both diluted 1:200 in blocking solution for 2 h at room temperature. After washing with PBS-T the blots were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (EuroClone, Milano, Italy) diluted 1:100,000 in blocking solution for 1 h at room temperature. After further washing with PBS-T, immunoreactive bands were visualized using luminol reagents and Hyperfilm-ECL film (Amersham Biosciences, Europe, GMBH) accordingly to the manufacturer's instructions. To normalize the bands, filters were stripped and re-probed with a mouse anti-α-tubulin. Bands density were quantified densitometrically.

4.2.6. Actin labeling

After incubation with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24, COBs were rinsed with 0.1 M phosphate-buffered saline solution (PBS), pH 7.4, and fixed with 4% paraformaldehyde (PFA) diluted in PBS for 25 min at room temperature. Cells were permeabilized with 0.3% Triton X-100 for 20 min on ice. Then, COBs were incubated with 4 × 10^{-6} mol/L phalloidin tetramethylrhodamine isothiocyanate (TRITC) conjugate (Sigma Aldrich, Milano Italy) for 20 min at room temperature. After rinsing in PBS, coverslips were mounted on slides with PBS/glycerol (1/1) [28]. Slides were imaged using a Leica DM 2500 fluorescent microscopy.

4.2.7. Toluidine blue–hematoxylin/eosin

COBs were treated with compounds **5**, **8**, **13** (10^{-6} M) and IDMC (10^{-6} M) for 24. Then, cultures were rinsed with 0.1 M PBS, pH 7.4, and fixed with 4% PFA diluted in PBS for 25 min at room temperature. After further washing in PBS, cells were stained with toluidine blue (Sigma–Aldrich, Milano, Italy) or with hematoxylin/eosin (Sigma–Aldrich, Milano, Italy); then, coverslips were mounted on slides with mounting medium and were monitored by a Leica DM 2500 optical microscopy.

Acknowledgments

We thank Sapienza University of Rome (Ateneo Project 2010 "nuovi pirroli funzionalizzati con anti-infiammatori per la realizzazione di nanoparticelle bioattive") and University of Camerino for the financial supports (Ateneo funds FAR 2010).

References

[1] J.A. Mitchell, P. Akarasereenont, C. Thiemermann, R.J. Flower, J.R. Vane, Proc. Nat. Acad. Sci. U. S. A. 90 (1993) 11693–11697.
[2] Z. Zhan, G. Huang, J. Nanotechnol. (2012) 1–12. http://dx.doi.org/10.1155/2012/748909.
[3] H. DeLeon, T.D. Nguyen, M. Nar, N.A. D'Souza, T.D. Golden, Mat. Chem. Phys. 132 (2012) 409–415.
[4] K.M. Manjanna, B. Shivakumar, T.M. Pramod Kumar, Crit. Rev. Ther. Drug Carrier Syst. 27 (2010) 509–545.
[5] F. Canal, J. Sanchis, M.J. Vicent, Curr. Opin. Biotechnol. 22 (2011) 894–900.
[6] J. Serra Moreno, S. Panero, S. Materazzi, A. Martinelli, M.G. Sabbieti, D. Agas, G. Materazzi, J. Biomed. Mater. Res. Part A 88 (2009) 832–840.
[7] X. Luo, C. Matranga, S. Tan, N. Alba, X.T. Cui, Biomaterials 32 (2011) 6316–6323.
[8] B.C. Thompson, R.T. Richardson, S.E. Moulton, A.J. Evans, S. O'Leary, G.M. Clark, G.G. Wallace, J. Control. Release 141 (2010) 161–167.
[9] C. Zonta, F. Fabris, O. De Lucchi, Org. Lett. 7 (2005) 1003–1006.
[10] M. Kakushima, P. Hamel, R. Frenette, J. Rokach, J. Org. Chem. 48 (1983) 3214–3219.
[11] N. Costantini, G. Wegner, M. Mierzwa, T. Pakula, Macromol. Chem. Phys. 206 (2005) 1345–1354.
[12] S. Khanna, M. Madan, A. Vangoori, R. Banerjee, R. Thaimattam, S. Jafar Sadik Basha, M. Ramesh, S.R. Casturi, M. Pal, Bioorg. Med. Chem. 14 (2006) 4820–4833.
[13] E.A. John, P. Pollet, L. Gelbaum, J. Kubanek, J. Nat. Prod. 67 (2004) 1929–1931.
[14] B.L. Bray, P.H. Mathies, R. Naef, D.R. Solas, T.T. Tidwell, D.R. Artis, J.M. Muchowski, J. Org. Chem. 55 (1990) 6317–6328.
[15] G. Schwenker, K. Stiefvater, Arch. Pharm. 324 (1991) 547–550.
[16] P. Godillot, H. Korri-Youssoufi, P. Srivastava, A. El Kassmi, F. Garnier, Synth. Met. 83 (1996) 117–123.
[17] H.Q.A. Lê, H. Sauriat-Dorizon, H. Korri-Youssoufi, Anal. Chim. Acta 674 (2010) 1–8.
[18] S.J. Wey, M.E. Augustyniak, E.D. Cochran, J.L. Ellis, X. Fang, D.S. Garvey, D.R. Janero, L.G. Letts, A.M. Martino, T.L. Melim, J. Med. Chem. 50 (2007) 6367–6382.
[19] A.S. Kalgutkar, B.C. Crews, S. Saleh, D. Prudhomme, L.J. Marnett, Bioorg. Med. Chem. 13 (2005) 6810–6822.
[20] H. Wang, J. Wei, C. Yang, H. Zhao, D. Li, Z. Yin, Z. Yang, Biomaterials 33 (2012) 5848–5853.
[21] K. Kaewprapan, P. Inprakhon, E. Marie, A. Durand, Carbohydr. Polym. 88 (2012) 875–881.
[22] G. Leriche, L. Chisholm, A. Wagner, Bioorg. Med. Chem. 20 (2012) 571–582.
[23] E. Jimi, I. Nakamura, H. Amano, Y. Taguchi, T. Tsurukai, M. Tamura, N. Takahashi, T. Suda, Endocrinology 137 (1996) 2187–2190.
[24] P. Godillot, H. Korri-Youssoufi, P. Srivastava, A. El Kassmi, F. Garnier, Synth. Met. 83 (1997) 117–123.
[25] A. Montero, Y. Okada, M. Tomita, I. Masato, H. Tsurukami, T. Nakamura, T. Doetschman, J.D. Coffin, M.M. Hurley, J. Clin. Invest. 105 (2000) 1085–1093.
[26] M.G. Sabbieti, D. Agas, L. Marchetti, G. Santoni, C. Amantini, L. Xiao, G. Manghi, M.M. Hurley, J. Cell. Physiol. 224 (2010) 465–474.
[27] D. Agas, L. Marchetti, G. Menghi, S. Materazzi, G. Materazzi, M. Capacchietti, M.M. Hurley, M.G. Sabbieti, J. Cell. Physiol. 241 (2008) 145–152.
[28] L. Marchetti, M.G. Sabbieti, M. Menghi, S. Materazzi, M.M. Hurley, G. Menghi, Histol. Histopathol. 17 (2002) 1061–1066.