Food and Chemical Toxicology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Structure–toxicity relationship and structure–activity relationship study of 2-phenylaminophenylacetic acid derived compounds

Yi Yun Pang [a], Wee Kiang Yeo [a], Kep Yong Loh [b], Mei Lin Go [a], Han Kiat Ho [a,*]

[a] Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
[b] National Cancer Centre, 11 Hospital Drive, Singapore 169610, Singapore

ARTICLE INFO

Article history:
Received 25 September 2013
Accepted 12 June 2014
Available online xxxx

Keywords:
Diclofenac
Lumiracoxib
Liver
Structure–toxicity relationship
Structure–activity-relationship

ABSTRACT

2-Phenylaminophenylacetic acid is a widely-exploited chemical scaffold whereby notable NSAIDs such as diclofenac and lumiracoxib were derived. Yet, their clinical usage has been associated with toxicities in the liver. While some studies have attributed toxicities to the bioactivation of both drugs to reactive intermediates, the structural predisposition for toxicity, as well as relationship between this toxicity and COX inhibitory activity has not been elucidated. In this study, we aimed to address their intricate link by synthesizing compounds that possess the 2-phenylaminophenylacetic acid backbone with varying alkyl and halogen substituents at three positions critical to the COX inhibitory pharmacophore. These compounds were subjected to cytotoxicity testing on two liver cell lines of contrasting metabolic competencies. We observed higher toxicity in the more metabolically competent cell line, supporting the role of bioactivation as a prerequisite for toxicity. We have also shown that structural changes on the chemical scaffold exerted pronounced effect on liver cytotoxicity. The most lipophilic and brominated compound (24) was identified as the most cytotoxic of all the compounds. A concurrent determination of their pharmacological activity using COX inhibition assays allowed us to derive a safety profile, which showed that selectivity towards COX-2 negatively affected activity and toxicity.

1. Introduction

2-Phenylaminophenylacetic acid is a simple but robust scaffold that has been explored widely over the years, producing two non-steroidal inflammatory drugs (NSAIDs), namely diclofenac (Voltaren®, {2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid) and lumiracoxib (Prexige®, {2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl}acetic acid). Despite their structural similarity, diclofenac and lumiracoxib are associated with vastly different pharmacological and toxicological profiles. Lumiracoxib specifically inhibits the cyclooxygenase-2 (COX-2) enzyme whereas diclofenac inhibits both isoforms (COX-1, COX-2) of the enzyme. In addition, both drugs are known to cause liver injury, albeit to different degrees. Nearly one-third of reported NSAID-induced hepatotoxicities were attributed to diclofenac (Agundez et al., 2011). In spite of its prevalence, diclofenac is still widely used because only a very small percentage of the liver toxicities are fatal, requiring hospitalization or liver transplants (Bessone, 2010). On the other hand, several cases of life threatening hepatotoxicities have been reported for lumiracoxib shortly after its approval and this has resulted in its withdrawal from clinical use in several countries (United-Nations, 2009). Therefore, even though 2-phenylaminophenylacetic acid analogs continue to present opportunities as an important scaffold for the development of other potent anti-inflammatory agents, their corresponding toxicological effects would need to be mitigated.

Collective efforts by independent groups have provided important structural insights into the pharmacological activity of 2-phenylaminophenylacetic acids. It has been shown that the methyl group at R_1 on ring A (Fig. 1) conferred selectivity to COX-2 for lumiracoxib (Blobaum and Marnett, 2007). The substituents at R_2 and R_3 on ring B (Fig. 1) were shown to influence efficacy of the compound through affecting the angle of twist between the two rings which determines affinity for the active site of the COX enzyme (Moser et al., 1990). Larger *ortho* substituents such

Abbreviations: (±)-BINAP, (±)-2,2′-bis(diphenylphosphino)-1,1′-binaphthalene; cLogP, calculated partition coefficient; COX, cyclooxygenase; CYP, cytochrome P450 for a specific isoform; DCM, dichloromethane; ELISA, enzyme-linked immunosorbent assay; EtOAc, ethyl acetate; IC_{50}, median inhibitory concentration; LC_{50}, median lethal concentration; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSAIDs, non-steroidal inflammatory drugs; PGE_2, prostaglandin-E_2; RLU, relative light units; TXB_2, Thromboxane-B_2.

* Corresponding author. Tel.: +65 6516 7963; fax: +65 67791554.
E-mail address: phahohk@nus.edu.sg (H.K. Ho).

http://dx.doi.org/10.1016/j.fct.2014.06.013
0278-6915/

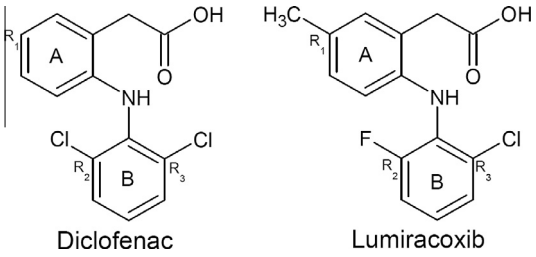

Fig. 1. Structures of diclofenac and lumiracoxib. Lumiracoxib differs from diclofenac by a methyl substituent at R_1 on ring A and a fluorine substituent at R_2 on ring B.

as chlorine and methyl were shown to affect the angle of twist more favorably. While the impact of these substituents on COX inhibition has been extensively characterized, much less is known about their effects on toxicity. To date, the toxicities of diclofenac and lumiracoxib have been linked to the formation of reactive metabolites in the liver via Phase I and Phase II metabolism. The bioactivation of diclofenac to reactive quinone imines, arene oxides and 1-β-O-acyl glucuronides were at least in part, responsible for the observed hepatotoxicity (Kretzrommel and Boelsterli, 1993, 1994; Leemann et al., 1993; Shen et al., 1999; Tang et al., 1999; Poon et al., 2001). *In vitro* bioactivation studies on lumiracoxib suggested a similar bioactivation process as that of diclofenac. 4′-Hydroxy-lumiracoxib was detected and was shown to further oxidize to a quinone imine (Li et al., 2008). Formation of an imine methide trapped as 5-methyl-glutathione-lumiracoxib was also detected (Kang et al., 2009). While these earlier studies postulated some plausible bioactivation pathways related to the toxicities of the two drugs, the question remains as to how subtle changes in the functional groups (methyl vs. hydrogen and chloro vs. fluoro) of diclofenac and lumiracoxib could have resulted in such disparate levels of toxicities. More importantly, there is limited understanding of the structural trends influencing toxicity within this scaffold, in the absence of which, effective approaches to achieve an optimal activity-toxicity balance would be difficult.

Therefore, in this study, we systematically synthesized analogs of diclofenac and lumiracoxib with the objective of deriving a qualitative structure–toxicity relationship. Test compounds were tested for their cytotoxicities in liver cell lines of differing metabolic competencies in order to expose any bioactivation-dependent toxicity. Accordingly, we have shown that structural changes at R_1 of ring A and R_2 and R_3 on ring B affected liver cytotoxicity, with the brominated compound **24** being the most toxic compound. Toxicity was shown to increase with increasing size of the alkyl and halogen substituents. Comparison of lipophilicity and toxicity data allowed us to propose that lipophilicity played a plausible role in the cytotoxicity results obtained. A safety profile was generated for the compounds using both *in vitro* toxicity and activity data, which showed that selectivity towards COX-2 negatively affected activity and toxicity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) and soybean trypsin inhibitor were obtained from Gibco® (Life Technologies, Carlsbad, CA). ITS Universal Culture Supplement Premix containing 5 mg/mL insulin, 5 mg/mL transferrin, 5 ng/mL selenium was obtained from BD Bioscience (Belford, MA). Fetal bovine serum (FBS) was obtained from Hyclone (Thermo Scientific, Waltharm, MA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Duchefa Biochemie (Haarlem, Netherlands). Luciferin-H and Luciferin-PFBE P450-Glo assay kits were obtained from Promega (Fitchburg, WI). Thromboxane-B_2 (TXB_2) and prostaglandin-E_2 (PGE_2) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cayman Chemical Company (Ann Arbor, MI). HPLC/spectro grade acetonitrile and methanol were purchased from Tedia (Fairfield, OH). All other chemicals and reagents were obtained from Sigma Aldrich (St. Louis, MO) or Alfa Aesar (Ward Hill, MA) unless otherwise mentioned.

2.2. Synthesis

Compounds **1**, **2** and **4** were synthesized via Scheme 1. Compounds **5**–**8** were synethsized via Scheme 2. The remaining compounds **9**–**24** were synthesized via Scheme 3. Merck silica 60 F254 sheets and Merck silica gel (0.040–0.063 mm) (Merck KGaA, Darmstadt, Germany) were used for thin layer chromatography (TLC) and flash chromatography respectively. Final compounds characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DPX 300 or Bruker ADVIII 400 spectrometer (Billerica, MA). Chemical shifts were reported in parts per million (ppm, δ) and referenced to residual solvents, deuterated chloroform ($CDCl_3$) (δ 7.260) or deuterated dimethyl sulfoxide (DMSO-d_6) (δ 2.500) for ^1H spectra and DMSO-d_6 (δ 39.43) for proton decoupled ^{13}C NMR. Coupling constants (*J*) were reported in Hertz (Hz). Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), sextet (sext), septet (sept), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), triplet of doublets (td), triplet of triplets (tt) and multiplet (m). Mass spectra were captured on a Sciex API 3000 Qtrap (AB Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) probe. Masses of the compounds were reported in *m/z* values for the molecular ion detected. Melting point was acquired on a Gallenkamp melting point apparatus (Weiss Technik UK, United Kingdom). All characterization data of intermediates and final compounds are listed in the Supplementary information.

2.2.1. Extraction of diclofenac (3) from Voltaren tablets

The enteric coating of six Voltaren® tablets (50 mg diclofenac per tablet, Norvatis, Switzerland) was removed. The tablets were then powdered with a pestle and mortar. To the powdered tablets (1.66 g), methanol (30 mL) and glacial acetic acid (1 mL) were added and the mixture sonicated for 5 min. The mixture was then shaken gently for 1 min and filtered. Water (30 mL) was added to the filtrate and the residue was removed by filtration under reduced pressure, after which it was washed with cold water (5 mL × 4). The residue (**A3**) was allowed to dry in an oven (50 °C) overnight.

2.2.2. Synthesis of compounds *1*, *2* and *4* (Scheme 1)

2.2.2.1. Synthesis of 2-iodophenyl-N, N-dimethylacetamide (25). The method reported by Kenny et al. (2004) was followed with some modifications. 2-Iodophenylacetic acid (0.53 g, 1.98 mmol) was dissolved in 12 mL of dichloromethane (DCM). To this solution, thionyl chloride (0.5 mL, 6.88 mmol) was added drop-wise with stirring at 0 °C. DMF (1 drop) was added and the solution stirred at room temperature for 2 h, then evaporated to dryness. DCM was added, the mixture evaporated and the evaporating procedure was repeated again. The product was re-dissolved in DCM (5 mL) and added drop-wise to a two-phase system of 40% w/w aqueous dimethylamine (12 mL) in water (35 mL) and DCM (50 mL), which was stirred vigorously at 0 °C. After 1 h, the organic layer was separated, washed with 1 M HCl, 10% aqueous Na_2CO_3, and water, and dried over anhydrous Na_2SO_4 before evaporating to dryness. Purification by chromatography (eluting with ethyl acetate (EtOAc): hexane, 1:3) afforded **25** as a colorless oil.

*2.2.2.2. General procedure for the synthesis of 2-[(2,6-disubstituted phenyl)amino)phenyl-N,N,-diethylacetamides (**26**–**28**).* A mixture of **25** (0.24 g, 0.83 mmol), 2,6-disubstituted aniline (1.90 mmol), anhydrous K_2CO_3 (0.11 g, 0.76 mmol), CuI (0.011 g, 0.052 mmol) and freshly activated Cu (Vogel, 1989) (0.035 g) in toluene (5 mL) in a Dean–Stark apparatus filled with 4 Å molecular sieves was stirred and refluxed for 96 h. The mixture was filtered while still warm over celite, concentrated, and re-dissolved in EtOAc, then washed with water, dried over anhydrous Na_2SO_4 and evaporated. Purification by chromatography (eluting with EtOAc:hexane, 1:4 followed by 2:3) afforded the product as crystals.

*2.2.2.3. General procedure for hydrolysis of acetamide to free acid (**1**, **2** and **4**).* The 2-[(2,6-disubstituted phenyl)amino)phenyl-N,N,-diethylacetamide (**26**–**28**) (0.16 M) was dissolved in ethanolic KOH (0.67 M) and refluxed overnight in an inert N_2 atmosphere. The solution was cooled to room temperature, concentrated and then re-dissolved in water (10 mL). The aqueous solution was extracted with EtOAc (2 × 30 mL) and the organic layer discarded. The aqueous solution was acidified with 1 M HCl to pH 3 and extracted with EtOAc (2 × 30 mL), washed with water, dried over Na_2SO_4 and evaporated to dryness. Purification by chromatography (eluting with EtOAc:hexane/1% acetic acid, 1:4 to 2:3), followed by recrystallization from EtOAc/hexane afforded the product as powder.

2.2.3. Synthesis of compounds *5*–*8* (Scheme 2)

*2.2.3.1. General procedure for the syntheses of 2,6-disubstituted-N-(p-alkyl)anilines (**29**–**48**).* The reaction was carried out as previously reported (Wolfe and Buchwald, 2000). Toluene (2 mL) and (±)-2,2′-bis(diphenylphosphino)-1,1′-binaphthalene ((±)-BINAP) (9.3 mg, 1.5 mol%) were stirred under argon at 80 °C for 1 min. $Pd(OAc)_2$ (2.2 mg, 1 mol%) was added to the mixture, purged with argon for