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Microwave-assisted synthesis of a series of 4,5-dihydro-1*H*--pyrazoles endowed with selective COX-1 inhibitory potency

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Abstract: Considerable efforts have been directed towards the discovery of selective cyclooxygenase isoxyme 1 (COX-1) inhibitors due to the recent work highlighting the involvement of COX-1 in the pathogenesis of pain, neuroinflammation, cancer and cardiovascular disorders. In this context, this paper aims to describe 2-pyrazolines endowed with selective COX-1 inhibitory potency. An efficient microwave-assisted synthetic method was applied for the preparation of a series of pyrazolines, which were tested for their COX-1 and cyclooxygenase isoxyme 2 (COX-2) inhibitory effects using a colorimetric assay. The cytotoxic properties of the most potent derivatives on NIH/3T3 fibroblast cells were determined using MTT method. 1-(3-Fluorophenyl)-5-(3,4-methylendioxyphenyl)-3-(2-thienyl)-4,5-dihydro-1*H*-pyrazole (**2g**) and 1-(3-bromophenyl)-5-(3,4-methylendioxyphenyl)-3-(2-thienyl)-4,5-dihydro-1*H*-pyrazole (**2h**) were determined as selective COX-1 inhibitors. According to the *in silico* data obtained from Schrödinger's QikProp module, both compounds are estimated to possess favourable oral bioavailability and drug-likeness. This work could be a rational guideline for further modifications at different sites on 2-pyrazoline motif to bring out a new class of selective COX-1 inhibitors.

Keywords: pyrazoline; microwave heating; cyclooxygenase-1 inhibition.

INTRODUCTION

Prostanoids (prostaglandins (PGs), thromboxane and prostacyclin) belong to the eicosanoid family of lipid mediators generated from arachidonic acid (AA).[1,2] Prostanoids play a central role in numerous physiological (*e.g.*, gastrointestinal (GI) integrity) and pathological (*e.g.* inflammation) processes.[2,3]

Cyclooxygenase (COX) is a rate-limiting enzyme implicated in the conversion of AA into prostanoids. There are two COX isozymes, namely the constitutive COX-1 and the inducible COX-2.[2] Both COXs possess similar structures, catalytic

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features, and subcellular localizations[2] and yield the same product, prostaglandin H_2. However, COX isozymes differ in terms of expression, tissue distribution, and biological tasks.[4] COX-1 contributes to homeostasis in most tissues (*e.g.*, platelets, GI tract, kidney, brain, lung, liver and spleen) where it is expressed under normal physiological conditions for the synthesis of PGs and exerts cytoprotective action along with the regulation of platelet activity, gastric and renal functions.[4,5] In general, non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit both COX isoforms, cause GI damage through COX-1 inhibition.[6] In order to avoid GI toxicity, many researchers have focused on the discovery of selective COX-2 inhibitors based on the hypothesis that PGs beneficial for GI protection were generated solely by means of COX-1 activity, whilst PGs responsible for inflammation and pain were produced exclusively through COX-2 activity.[6,7] However, this assumption has lost its actuality since it is understood that COX-2 is constitutively expressed in some tissues and COX-1-derived PGs are also involved in inflammation and therefore COX-1 sparing is not adequate to prevent GI toxicity.[7,8]

Recent studies have revealed that COX-1 participates in the pathogenesis of many diseases (*e.g.*, cancer, neuroinflammation, cardiovascular diseases and pain).[4,9] It is noteworthy that low-dose aspirin is beneficial in the prevention of cardiovascular disorders through the inhibition of platelet COX-1. In addition, mounting evidence has also shown that long-term use of aspirin reduces the risk of some types of cancer and other diseases such as Alzheimer's disease.[9]

Despite all efforts devoted to the discovery of selective COX-1 inhibitors, there is only one selective COX-1 inhibitor (mofezolac) currently prescribed as a non-steroidal anti-inflammatory drug (NSAID) just in Japan for the management of pain/inflammation after surgery, trauma, or tooth extraction; lumbago, cervicobrachial syndrome and scapulohumeral periarthritis.[9,10] SC-560 and FR122047, selective COX-1 inhibitors commonly applied as reference agents in experimental studies, could not be introduced to the market as therapeutic agents because of their pharmacodynamic and pharmacokinetic drawbacks.[6,9]

From a chemical structural point of view, selective COX 1 inhibitors (mofezolac, SC-560 and FR122047, Fig. 1) possess a five-membered heteroaromatic central ring in common (isoxazole in mofezolac, pyrazole in SC-560, and thiazole in FR122047). Moreover, two aromatic rings linked to adjacent atoms of the five-membered heteroaromatic nucleus are found to be determinant.[6,9]

2-Pyrazoline (4,5-dihydro-1*H*-pyrazole), the partially reduced form of pyrazole, is a privileged member of the nitrogen-containing heterocycles due to its indispensable role in the discovery of new therapeutic drugs with improved potency and less toxicity along with favourable pharmacokinetic profiles.[11,12] 2--Pyrazolines have been reported to possess a broad range of biological activities (analgesic, anti-inflammatory, antitumor, antidepressant, antimicrobial, *etc*.)

known for their ability to interact with pivotal biological targets involved in diverse biochemical pathways.[11–27] There are many pyrazoline-based marketed agents as well as therapeutic candidates undergoing preclinical and clinical trials.[12] Some of them exert potent analgesic and anti-inflammatory action through the inhibition of COXs. Phenazone was the first pyrazoline-based agent used for the management of pain and inflammation. Dipyrone (metamizole, Fig. 2), was introduced to the market nearly a century ago and it is still used an analgesic and antipyretic drug in many countries worldwide.[27]

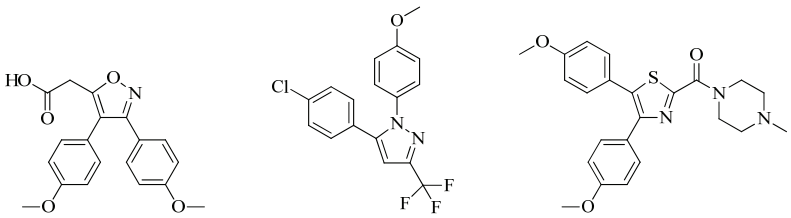


Fig. 1. Selective COX-1 inhibitors.

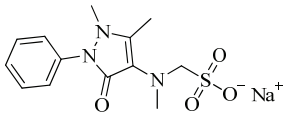


Fig. 2. Dipyrone.

A vast number of scientific reports related to 2-pyrazolines exerting marked COX inhibitory potency[17–27] prompted us to design a series of pyrazoline-based selective COX-1 inhibitors. With the given aim, the microwave (MW)-assisted synthesis of eight 2-pyrazolines was carried out expeditiously and afterwards *in vitro* experiments were performed to evaluate their potency as selective COX-1 inhibitors.

EXPERIMENTAL

General procedure

The chemicals used without further purification were obtained from commercial vendors (Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Almanya), Acros Organics (Geel, Belgium), and Alfa Aesar (Karlsruhe, Germany). The reactions were performed in Monowave 400 MW reactor (Anton Paar, Graz, Austria) in sealed reaction vessels, power supply voltage: AC 230 V (±10%), 50 Hz/60 Hz, installed microwave power: 850 W, power consumption: 1600 V A, operating frequency: 2455 MHz. Compressed air cooling: 5.5 to 6 bar (80 to 87 psi). The reaction conditions are optimized by changing different temperatures and time under solvent medium. Melting points (M.p.) were detected by means of a digital melting point apparatus (Electrothermal, Staffordshire, UK) and are uncorrected. Thin layer chromatography (TLC) was performed on TLC silica gel 60 F254 aluminium sheets (Merck, Darmstadt, Germany) using petroleum ether-ethyl acetate solvent systems (3:1 and 1:1). IR spectra were recorded on a Fourier-transform IR spectrophotometer (Shimadzu, Tokyo, Japan). ^{1}H-

and ^{13}C-NMR spectra were acquired using a NMR spectrometer (at 300 and 75 MHz, respectively). (Bruker, Billerica, MA, USA). Chemical shifts were expressed in parts per million (ppm). HRMS spectra were recorded on a LCMS-IT-TOF system (Shimadzu, Kyoto, Japan). The spectral data of the compounds are given in Supplementary material to this paper.

Synthetic procedures

*General procedure for the preparation of 1-(2-thienyl)-3-(3,4-methylenedioxyphenyl)-2-propen-1-one (**1**)*

2-Acetylthiophene (0.02 mol) was reacted with piperonal (0.02 mol) in the presence of 40 % aqueous NaOH (5 mL) in absolute ethanol (30 mL) at room temperature (rt) for 24 h. Upon the completion of the reaction, the reaction mixture was poured into crushed ice. The precipitate was filtered, and washed with water. After drying, the product was recrystallized from ethanol.[28]

*General procedure for the preparation of 1-aryl-3-(2-thienyl)-5-(3,4-methylenedioxyphenyl)-2-pyrazolines (**2a**–**h**)*

A mixture of compound **1** (1 mmol) and arylhydrazine hydrochloride (1.5 mmol) in absolute ethanol (8 mL) was heated to 180 °C within 15 min and kept at this temperature for 18 min under MW irradiation in a reaction vial with magnetic stirring at 500 rpm in a Monowave 400 MW reactor equipped with a ruby thermometer. Upon the completion of the reaction, the reaction mixture was cooled to room temperature. The precipitate was collected by filtration and dried. The product was crystallized from ethanol.

Biochemistry

Determination of COX inhibitory activity. COX inhibitor screening assay (catalog No.: 701050) was performed to determine the inhibitory activities of compounds **2a**–**h** (at 100 μM) towards COX-1 and COX-2 according to the manufacturer's guideline (Cayman, Ann Arbor, MI, USA). All measurements were performed in triplicate and the results were expressed as mean ± *SD*. SC-560 (at 1 μM) was used as a selective COX-1 inhibitor, whereas drug rofecoxib (at 10 μM) was used as a selective COX-2 inhibitor.

Cell culture and drug treatment. NIH/3T3 mouse embriyonic fibroblast cells (ATCC® CRL-1658™) were cultured and drug treatments were performed as reported previously.[29]

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test. The level of cellular MTT (Sigma–Aldrich, St. Louis, MO, USA) reduction was quantified as explained earlier[30] with minor modifications.[29]

In silico *pharmacokinetic studies*. QikProp, an *in silico* absorption, distribution, metabolism, elimination (ADME) module within the Maestro suite produced by Schrödinger (Schrödinger Release 2022-2, LLC, New York, USA), was used to predict the crucial physicochemical parameters of compounds **2g** and **2h** for the assessment of their ADME profiles.

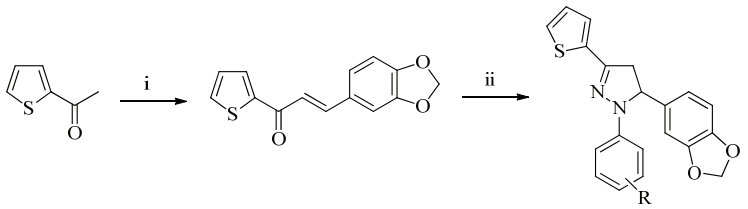
RESULTS AND DISCUSSION

Chemistry

The preparation of 2-pyrazolines (**2a**–**h**) followed the general pathway depicted in Scheme 1. The chalcone (**1**) was obtained *via* the Claisen–Schmidt condensation of 2-acetylthiophene with piperonal.

MW-assisted synthesis is recognized as an advantageous and eco-friendly technique for the rapid and efficient preparation of heterocyclic compounds inc-

luding nitrogen-containing heterocycles.[31–33] The employment of this technique in dedicated MW reactors provides efficient and controlled heating along with excellent parameter control and therefore MW heating has several advantages over conventional heating such as shortening reaction time and obtaining products with high purity and yield.[31–35] In this work, an efficient MW-assisted protocol was applied for the preparation of compounds **2a**–**h**. Compounds **2a**–**e** were previously synthesized by our research group using a conventional method.[28] The comparison between MW and conventional techniques was made by comparing yield and total reaction time (Table I). MW technique led to a reduction in reaction time (from 480 min to 18 min) and an increase in product yields.

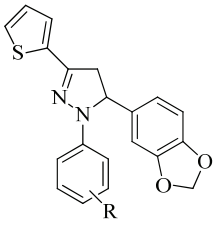


1 **2a-h**

R= 4-CN, 4-F, 4-Br, 4-CH_3, 4-SO_2CH_3, 3-NO_2, 3-F, 3-Br

Scheme 1. The synthetic route for the preparation of compounds **2a**–**h**. Reagents and conditions: i) piperonal, 40 % NaOH, absolute ethanol, rt, 24 h; ii) arylhydrazine hydrochloride, absolute ethanol, MW, 180 °C, 18 min.

TABLE I. MW technique vs conventional method for the preparation of the compounds

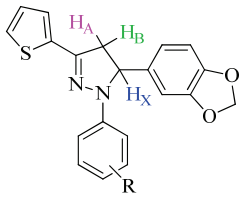


Compound	R	MW Irradiation		Conventional[a]	
		Yield, %	Time, min	Yield, %	Time, min
2a	4-CN	95	18	93	480
2b	4-F	82	18	76	480
2c	4-Br	91	18	90	480
2d	4-CH_3	73	18	47	480
2e	4-SO_2CH_3	94	18	63	480
2f	3-NO_2	84	18	–	–
2g	3-F	81	18	–	–
2h	3-Br	80	18	–	–

[a]Compounds **2a**–**e** were previously synthesized by our research team using a conventional method.[28] Compounds **2f**, **g** and **h** are reported for the first time in this work

The structures of compounds **2a**–**h** were confirmed by infrared (IR), nuclear magnetic resonance (NMR, ^{1}H and ^{13}C) and high resolution mass spectrometry (HRMS).

In the IR spectra of compounds **2a**–**h**, Fig. 3, the absence of a band at 1643.55 cm^{-1} due to the C=O stretching [28] confirmed that the formation of the 2-pyrazoline scaffold occurred efficiently. The C=N, C=C and C–N stretching bands appeared in the region 1612-1419 and 1396–1107 cm^{-1}, respectively. In the ^{1}H-NMR spectra of compounds **2a**–**h**, the CH_2 protons of the 2-pyrazoline scaffold resonated as a pair of doublet of doublets at 3.08–3.26 ppm (C_4-H_A) (J_{AB} = 17.28 to 17.64 Hz, J_{AX} 4.95–6.81 Hz) and 3.84–4.00 ppm (C_4-H_B) (J_{BA} 17.25–17.70 Hz, J_{BX} 11.91–12.06 Hz).



2a-h

R= 4-CN, 4-F, 4-Br, 4-CH_3, 4-SO_2CH_3, 3-NO_2, 3-F, 3-Br

Fig. 3. The ABX system of the pyrazoline scaffold belonging to compounds **2a**–**h**.

The CH proton appeared as a doublet of doublets at 5.36–5.63 ppm (C_5-H_X) (J_{BX} 11.85–12.06 Hz, J_{AX} 4.95–6.81 Hz). The O–CH_2–O protons gave rise to a singlet or a doublet in the region 5.97–6.04 ppm. The ^{13}C-NMR chemical shift values of the carbons at 44.14–44.63 ppm (C4), 62.53–64.11 ppm (C5) and 144.46–154.85 ppm (C3) supported the ^{1}H-NMR data confirming the formation of the pyrazoline motif. The HRMS data of compounds **2a**–**h** were also consistent with other spectral data.

Biochemistry

A colorimetric test was conducted to assess the inhibitory effects of compounds **2a**–**h** on COX-1 and COX-2 (Table II). Among compounds **2a**–**h**, compounds **2g** and **h** were found to selective COX-1 inhibitors as compared to SC--560. The inhibition of compounds **2g** and **h** at 100 μM for COX-1 were found as 50.92±2.80 and 57.70±2.64 %, respectively as compared to SC-560 (97.36± ±2.62 % at 1 μM), a selective COX-1 inhibitor. *m*-Fluoro and *m*-bromo substituents enhanced COX-1 inhibitory potency.

The replacement of the halogen atom with the nitro group (compound **2f**) led to the loss of COX-1 inhibitory potency. However, *m*-nitro substitution gave rise to selective COX-2 inhibitory activity (36.48±2.18 %). *p*-fluoro substitution (compound **2b**) caused the loss of inhibitory potency towards both COXs, whereas *p*-bromo substitution (compound **2c**) resulted in selective COX-2 inhi-

bitory potency (26.30±3.46 %). The inhibition of compounds **2c** and **f** at 100 μM were detected as 26.30±3.46 and 36.48±2.18 %, respectively in comparison with rofecoxib (98.36±1.86 % at 10 μM), which is a selective COX-2 inhibitor. Compounds **2a**, **b** and **e** did not show any inhibitory activity towards COXs. According to the results, *p*-cyano, *p*-fluoro and *p*-methylsulfonyl groups led to the loss of COX inhibitory activity.

TABLE II. The inhibitory effects of compounds **2a**–**h**, SC-560 and rofecoxib on COXs

Compound (100 μM)	Inhibition, %	
	COX-1	COX-2
2a	–	–
2b	–	–
2c	–	26.30±3.46
2d	9.65±1.56	–
2e	–	–
2f	–	36.48±2.18
2g	50.92±2.80	–
2h	57.70±2.64	–
SC-560 (1 μM)	97.36±2.62	–
Rofecoxib (10 μM)	–	98.36±1.86

Compounds **2g** and **h**, the most potent COX-1 inhibitors in this series, were subjected to MTT assay for the evaluation of their cytotoxicity towards NIH/3T3 (normal) cells. Both compounds did not exhibit any cytotoxicity towards NIH/ /3T3 cell line at the tested concentrations (IC_{50} > 500 μM).

In silico *ADME prediction*. *In silico* approaches are frequently used to assess pharmacokinetic profiles of drug candidates in drug development process since ADME experiments are not only costly and time-consuming for a vast number of chemicals, but also require a large number of animal tests and the corresponding ethical procedures.[36] In this context, a computational study for the prediction of the pharmacokinetic features of compounds **2g** and **h** was performed (Table III). The predicted values for total solvent accessible surface area (*SASA*), van der Waals surface area of polar nitrogen and oxygen atoms (*PSA*), octanol/water partition coefficient (*QP*log *Po*/*w*) and binding to human serum albumin (*QP*log *Khsa*) values were detected within the optimum range.

The predicted apparent Caco-2 cell permeability (*QPPCaco*) values of compounds **2g** and **2h** were found to be higher than 500 and therefore both compounds are estimated to possess good intestinal permeability. Compounds **2g** and **h** are also predicted to possess 100.000% human oral absorption. Furthermore, both compounds violated only one parameter of Lipinski's and Jorgensen's rules, making them drug-like molecules endowed with favourable oral bioavailability.

The capability of a drug to penetrate the blood-brain barrier (BBB) is required for its use in the treatment of central nervous system (CNS) dis-

orders.[38,39] Predicted brain/blood partition coefficient (*QP*log *BB*) was used to estimate the BBB permeability of each compound. The *QP*log *BB* values of both compounds were detected within the recommended values. Moreover, Madin–Darby canine kidney (MDCK) cell permeability is an additional criterion which is widely used for the assessment of BBB penetration.[40,41] The estimated apparent MDCK cell permeability (*QPPMDCK*) values of both compounds were found to be higher than 500. Based on the *in silico* data, compounds **2g** and **h** are predicted to possess the ability to cross BBB.

TABLE III. Predicted pharmacokinetic features of compounds **2g** and **h**

Property or descriptor	Compound **2g**	Compound **2h**	Range or recommended values
SASA	589.893	605.657	300.0–1000.0
PSA	32.674	32.687	7.0–200.0
*QP*log *Po*/*w*	5.704	6.041	–2.0–6.5
QPPCaco	8585.887	8883.859	<25 poor, >500 great
*QP*log *BB*	0.730	0.813	–3.0–1.2
QPPMDCK	10000.000	10000.000	<25 poor, >500 great
*QP*log *Khsa*	1.009	1.112	–1.5–1.5
Human oral absorption, %	100.000	100.000	>80 % is high, <25 % is poor
Rule of Five[a]	1	1	Maximum is 4
Rule of Three[b]	1	1	Maximum is 3

[a]Rule of Five: number of violations of Lipinski's rule of five. The rules are: molecular weight of the compound < 500, *QP*log *Po*/*w* < 5, hydrogen-bond donor atoms ≤ 5, hydrogen-bond acceptor atoms ≤ 10. Compounds that provide these rules are considered as drug-like molecules; [b]Rule of Three: number of violations of Jorgensen's rule of three. The three rules are: predicted aqueous solubility (*QP*log *S*) > –5.7, *QPPCaco* > 22 nm s^{-1}, primary metabolites < 7. Compounds with fewer (and preferably no) violations of these rules are more likely to be orally available agents[37]

CONCLUSION

In this paper was described an efficient MW-assisted protocol for the preparation of a series of pyrazolines (**2a**–**h**), which were investigated for their inhibitory effects on COXs at 100 μM using an *in vitro* colorimetric assay. According to *in vitro* experimental data, compounds **2g** and **h** were determined as selective COX-1 inhibitors in this series, as compared to SC-560. MTT test was applied to assess their cytotoxic effects on NIH/3T3 cells. None of the compounds displayed any cytotoxicity towards NIH/3T3 cell line at the tested concentrations. *In silico* ADME prediction was performed for the assessment of their pharmacokinetic features. Compounds **2g** and **h** are predicted to have favourable oral bioavailability and drug-likeness. Based on this work, a new generation of pyrazolines could be designed through the molecular modification of compounds **2g** and **h** for the treatment of many diseases in which selective COX-1 inhibition is required.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12058, or from the corresponding author on request.

ИЗВОД

МИКРОТАЛАСНА СИНТЕЗА СЕРИЈЕ ДЕРИВАТА 4,5-ДИХИДРО-1*H*-ПИРАЗОЛА КОЈИ ПОСЕДУЈУ ИЗРАЖЕНУ ИНХИБИТОРНУ АКТИВНОСТ ПРЕМА COX-1

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Учињен је значајан покушају проналажењу селективних COX-1 инхибитора током скоријих истраживања о значају инхибитора изозима 1 циклооксигеназе (COX-1) у патогенези бола, упале неурона, канцеру, и кардио-васкуларним поремећајима. У том правцу, у овом раду описани су деривати 2-пиразолина који поседују способност селективне инхибиције COX-1. Примењена је ефикасна метода микроталасне синтезе за добијање серије пиразолина који су тестирани као инхибитори COX-1 и инхибитора изоксима 2-циклооксигеназе (COX-2) применом колориметријске методе. Цитотоксична активност најактивнијих деривата је одређена на NIH/3T3 ћелијама фибропласта применом MTT метода. Показано је да су деривати 1-(3-флуорфенил)-5-(3,4-метиледиоксифенил)-3-(2-тиенил)-4,5-дихидро-1*H*-пиразол (**2g**) и 1-(3-бромфенил)-5-(3,4-метиледиоксифенил)-3-(2-тиенил)-4,5-дихидро-1*H*-пиразол (**2h**) селективни COX-1 инхибитори. На основу података добијених *in silico* прорачунима помоћу Schrödinger QikProp модула, процењено је да оба једињења имају повољну оралну биодоступност и повољне особине за примену као лек (drug-likeness). Овај рад би могао да буде основа за истраживање даљих модификација на различитим позицијама 2-пиразолинског језгра у циљу синтезе нове класе селективних COX-1 инхибитора.

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