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Regular paper

Synthesis, COX-1/2 inhibition and antioxidant activities of new oxicam analogues designed as potential chemopreventive agents*

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Oxicams (e.g. piroxicam, meloxicam) are widely used nonsteroidal anti-inflammatory drugs (NSAIDs). A large body of evidence from epidemiological and preclinical studies has shown that NSAIDs have a chemopreventive effect on different types of cancer, especially in colorectal cancer. Moreover, mounting evidence from preclinical and clinical studies suggests that persistent inflammation functions as a driving force in the journey to cancer. What is more, inflammation induces reactive oxygen and nitrogen species, which cause damage to important cellular components (e.g., DNA, proteins and lipids), which can directly or indirectly contribute to malignant cell transformation. In this study, we discuss the synthesis and the resultant newly synthesized oxicam derivatives which are potentially chemopreventive, and at the same time antioxidant. Compound 9c, with the highest therapeutic index in the LoVo cancer cell line, was found to be the most efficient in ROS scavenging activity under conditions of oxidative stress.

Key words: synthesis, chemoprevention, antioxidant, COX, NSAIDs, oxicams

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Abbreviations: NSAIDs non-steroidal anti-inflammatory drugs; COX cyclooxygenase; ROS reactive oxygen species; DMF dimethylformamide; TEA triethylamine; CNS central nervous system

INTRODUCTION

The term *chemoprevention* was introduced forty years ago by Michael B. Sporn, and defined as the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to an invasive disease (Sporn, 1976). It is very important to note that chemoprevention is not aimed at taking over the role of surgery and chemotherapy itself. It is rather a kind of adjuvant therapy involving perturbation of a variety of steps in tumor initiation, promotion and progression. Although a small number of published randomized trials have shown that some chemopreventive therapies may indeed be effective against cancer, there is still need to develop guidelines for management of patients with higher risk of cancer or early treatment with adjuvant chemoprevention therapy (Ulrich *et al.*, 2006; Rostom *et al.*, 2007; Harris *et al.*, 2008; Rothwell *et al.*, 2010 & 2011; Gravitz, 2011; Boghossian *et al.*, 2012; Cook *et al.*, 2013; Mansouri *et al.*, 2013; Sahin *et al.*, 2014).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been one of the most promising agents in chemoprevention, especially for colorectal cancer (Piazuelo *et al.*, 2015). They are widely used medications with a well–known molecular target. Their activity involves inhibition of the prostaglandin H_2 synthase (PGHS), also commonly called cyclooxygenase (COX). There are three known isoforms of the COX enzymes, the two most important are: COX-1 – the *constitutive* form, and COX-2 – the *inducible* form of the enzyme (Vane *et al.*, 1998). COX-3 is a poorly understood isoform of COX occurring mainly in the CNS (Dhingra *et al.*, 2014).

In the last two decades a lot of research results showing the connection between the overexpression of COX-2 and occurrence of many human malignancies, e.g. colorectal, breast, pancreatic and lung cancer, were published (Gupta *et al.*, 2001; Ghosh *et al.*, 2010). The data revealed that COX-2 plays a role in different stages of cancer progression, as well as metastasis formation (Lee *et al.*, 2007). Targeting COX-2 is one of the recent therapeutic methods for the treatment of many types of cancer (Dannenberg *et al.*, 2001; Al-Fayez *et al.*, 2006).

NSAIDs are mainly used to treat pain, arthritis and fever. Low-dose of aspirin therapy (75 mg per day) has also proven to be effective in reducing the risk of stroke and heart attack, but recently inhibition of COX-1 causing inhibition of platelet activation, facilitation of immunosurveillance and prevention of haematogenous spread of malignancy has been suggested as another putative mechanism of cancer prevention (Thun *et al.*, 2012). Nevertheless, the main NSAID anticancer mechanism is thought to be the decrease of synthesis of prostaglandin E_2 *via* COX-2 inhibition which causes a decrease in tumor cell proliferation and angiogenesis, and increase in apoptosis. Although many of the NSAID anticancer mechanisms are defined as COX dependent, several signal transduction pathways (including for example nuclear factor-kappa B) have been confirmed as NSAIDs' COX independent way of acting (Greenspan *et al.*, 2011; Liao *et al.*, 2012). The correlation between the COX enzyme expression and cancer, together with prognosis factors and putative chemopreventive agents, have been widely studied and reviewed in the literature (Martin *et al.*, 2013; Park *et al.*, 2014).

We designed and synthesized new compounds with potential chemopreventive activity. The lead structure for the synthesis was oxicam scaffold. Oxicams (e.g. piroxicam, meloxicam) belong to the NSAID group. As

oxicam analogues, our new compounds were believed to possess an analgesic and anti-inflammatory activity via COX inhibition. Their cytotoxicity, as well as antioxidant properties, were also examined because an additional benefit expected in chemoprevention of cancer is ROS scavenging activity (Romero-Canelón *et al.*, 2015).

MATERIALS AND METHODS

Chemicals

Sulforhodamine (SRB), Trizma-base and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), 2 mM L-glutamine solution, antibiotic solutions: penicillin (100 U/mL) and streptomycin (0.1 mg/mL), and trypsin EDTA solution were obtained from Lonza (Verviers, Belgium). Cell culture plastic flasks (75 cm^2), as well as 96-wells culture plastic plates, were purchased from Lonza (Verviers, Belgium). Phosphate buffered saline (PBS) and 0.4% trypan blue solution were obtained from POCH (Gliwice, Poland). All other chemicals used in this study were of analytical grade.

Synthesis

Unless otherwise noted, chemicals and reagents were obtained from commercial suppliers and used without further purification. Dry solvents were obtained according to the standard procedure. All reactions were monitored by thin layer chromatography on silica gel 60 F254-coated TLC plates (Fluka Chemie GmbH) and visualized by UV light at 254 nm. Flash column chromatography on silica gel column (230–400 mesh) was used to purify the final product. Melting points were recorded using open capillary tubes and are uncorrected. The proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Bruker 300 MHz NMR spectrometer using $CDCl_3$ or DMSO-d_6 as solvents and tetramethylsilane (TMS) as an internal reference. Chemical shifts are reported in parts per million (ppm). Signal multiplicities were characterized as: s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), quin (quintet) and m (multiplet). Infrared spectra were recorded on a Perkin-Elmer Spectrum Two UATR FT-IR spectrometer, and frequencies are reported in cm^{-1}. Samples were applied as solids. Elemental analyses for carbon, hydrogen and nitrogen were carried out on a Carlo Erba NA 1500 analyzer and were within ±0.4% of the theoretical value.

General procedure for the preparation of *2-[3-(4-arylpiperazinyl)propyl]-4-hydroxy-3-(4-substituted-benzoyl)-2H-1,2-benzothiazine 1,1-dioxides 8a-i and 2-[2-(4-arylpiperazinyl)-2-oxoethyl]-4-hydroxy-3-(4-substituted-benzoyl)-2H-1,2-benzothiazine 1,1-dioxides* **9a-g** (Scheme 1).

Synthesis and experimental data for the *1,1-dioxo-2-[2-oxo-2-(p-substituted-phenyl)ethyl]-1,2-benzothiazol-3-one* **3a-d** compounds were previously reported (Krzyżak *et al.*, 2014; Szczęśniak-Sięga *et al.*, 2014; Yoshimura *et al.*, 2015).

Synthesis of *1,1-dioxo-3-(p-substituted-benzoyl)-4-hydroxy-2H-1,2-benzothiazines* **4a-d**

Synthesis and experimental data of compounds **4a**, **4b** and **4d** were previously reported (Krzyżak *et al.*, 2014; Szczęśniak-Sięga *et al.*, 2014).

Synthesis of *1,1-dioxo-3-(p-methylbenzoyl)-4-hydroxy-2H-1,2-benzothiazine* **4c**

Three millimoles of **3c** were dissolved in 7.5 ml sodium ethoxide (0.23 g of Na in 10 ml of anhydrous ethanol) at 40ºC and stirred with heating to 55–60ºC for 15 min. Color changes from white to deep red were observed. After dissolving all of the substance, the mixture was rapidly cooled to 25ºC and 7.5 ml of HCl (9%) were added. The color changed from deep red to deep yellow and the product precipitated. The solid was filtered off, dried, and purified by crystallization from EtOH to give **4c** with 85% yield.

1,1-dioxo-3-(p-methylbenzoyl)-4-hydroxy-2H-1,2-benzothiazine **4c** 85% yield, mp 232–233ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.47 (s, 3H, CH_3), 5.83 (s, 1H, NH), 7.28-8.27 (m, 8H, ArH), 15.90 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 3242 (NH), 1589 (C=O), 1369, 1177 (SO_2). Anal. Calcd for $C_{16}H_{13}NO_4S$ (315.34): C, 60.94; H, 4.16; N, 4.44; Found: C, 60.88; H, 4.19; N, 4.36.

Synthesis and experimental data for the *1-(3-chloropropyl)-4-arylpiperazine* **5a-d** compounds were previously reported (Lopez *et al.*, 2010; Krzyżak *et al.*, 2014; Maniewska *et al.*, 2014; Paudel *et al.*, 2016).

Synthesis and experimental data for the *1-(2-chloro-1-oxoethyl)-4-arylpiperazine* **6a-c** compounds were previously reported (Szczęśniak-Sięga *et al.*, 2014; Muszalska *et al.*, 2015).

Synthesis and experimental data for the *2-(2-chloro-1-oxoethyl)-1,3,4,9-tetrahydro-β-carboline* **7** compound was previously reported (Zhou *et al.*, 2016).

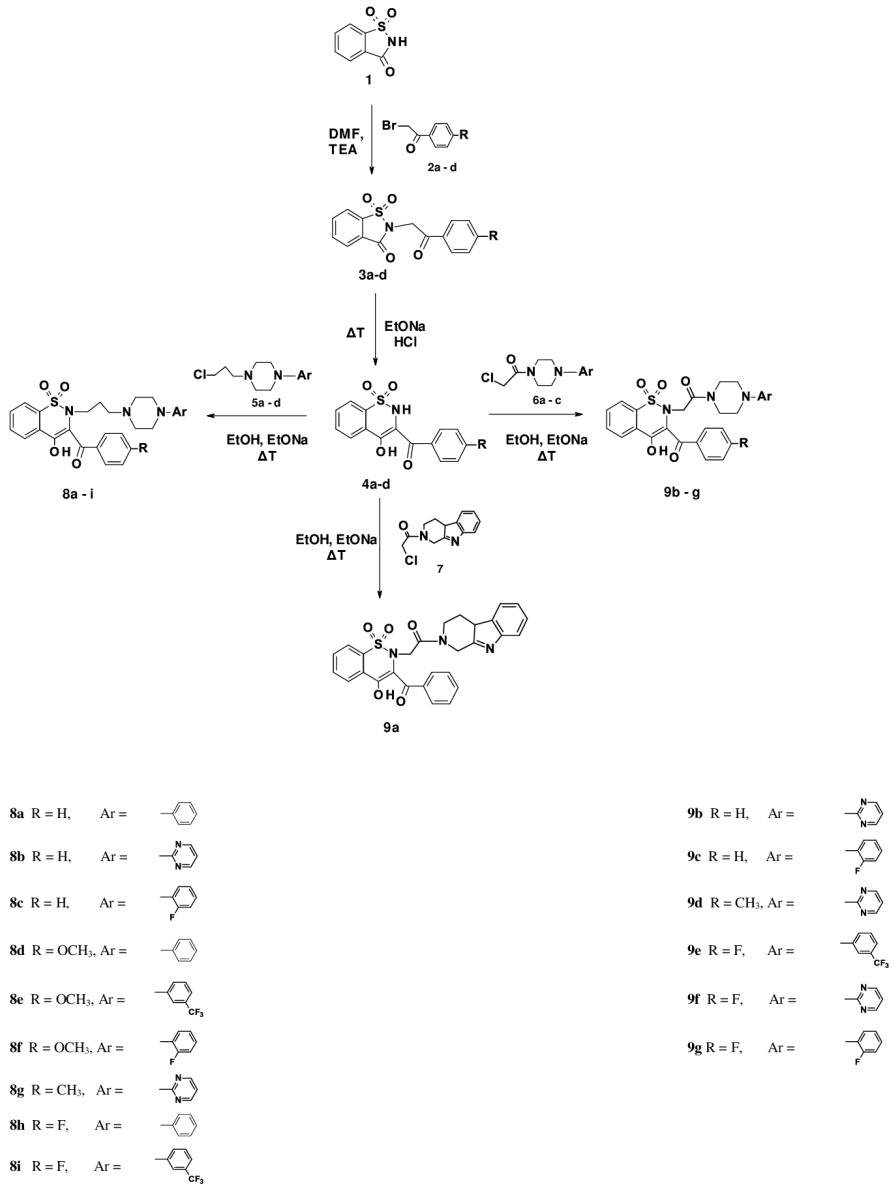
Synthesis of *2-[3-(4-arylpiperazinyl)propyl]-4-hydroxy-3-(4-substituted-benzoyl)-2H-1,2-benzothiazine 1,1-dioxides* **8a-i**

To the stirred mixture of 5 mmol of corresponding 1,2-benzothiazines **4a-d** in 20 ml of anhydrous EtOH was added to 5 ml of EtONa (0.115 g of Na in 5 ml of anhydrous ethanol). Then, 5 mmol of corresponding 1-(3-chloropropyl)-4-arylpiperazine **5a-d** were added and refluxed with stirring for 10 h. When the reaction ended, which was monitored on TLC plates, ethanol was distilled off, the residue was treated with 50 ml of $CHCl_3$ and the insoluble materials were filtered off. The filtrate was then evaporated and the residue was purified by crystallization from EtOH to give desirable products **8a-i**. Compounds **8a**, **8d** and **8h** were previously described (Krzyżak *et al.*, 2014).

3-benzoyl-4-hydroxy-2-{3-[4-(2-pyrimidyl)-1-piperazinyl]propyl}-2H-1,2-benzothiazine 1,1-dioxide **8b** 41% yield, mp 173–176ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.20 (brs, 2H, $CH_2C\mathbf{H_2}CH_2$), 1.93 (s, 2H, $CH_2CH_2C\mathbf{H_2}$-$N_{piperazine}$), 2.11 (s, 4H, $H_{piperazine}$), 3.17 (brs, 2H, $C\mathbf{H_2}CH_2CH_2$), 3.64-3.71 (brs, 4H, $H_{piperazine}$), 6.44-6.47 [t, *J*=6 Hz, 1H, $CH(5)_{pyrimidine}$], 7.48-8.21 (m, 9H, ArH), 8.26-8.27 [d, *J*=6 Hz, 2H, $CH(4\text{ and }6)_{pyrimidine}$], 15.75 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 1618 (C=O), 1346, 1178 (SO_2). Anal. Calcd for $C_{26}H_{27}N_5O_4S$ (505.59): C, 61.77; H, 5.38; N, 13.85; Found: C, 61.69; H, 5.18; N, 13.78.

3-benzoyl-2-{3-[4-(2-fluorophenyl)-1-piperazinyl]propyl}-4-hydroxy-2H-1,2-benzothiazine 1,1-dioxide **8c** 44% yield, mp 147–149ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.24 (brs, 2H, $CH_2C\mathbf{H_2}CH_2$), 1.96 (brs, 2H, CH_2CH_2-$C\mathbf{H_2}N_{piperazine}$), 2.26 (brs, 4H, $H_{piperazine}$), 2.94-3.60 [m, 6H, $C\mathbf{H_2}N(C\mathbf{H_2})_2$], 6.86-8.21 (m, 13H, ArH), 15.60 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 1615 (C=O), 1329, 1177 (SO_2). Anal. Calcd for $C_{28}H_{28}FN_3O_4S$ (521.60): C, 64.47; H, 5.41; N, 8.06; Found: C, 64.67; H, 5.35; N, 8.05.

4-hydroxy-3-(4-methoxybenzoyl)-2-{3-[4-(3-trifluoromethylphenyl)-1-piperazinyl]propyl}-2H-1,2-benzothiazine 1,1-dioxide **8e** 46% yield, mp 121–123ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.24 (brs, 2H, $CH_2C\mathbf{H_2}CH_2$), 2.02 (brs, 2H, $CH_2CH_2C\mathbf{H_2}N_{piperazine}$), 2.28 (brs, 4H, $H_{piperazine}$), 3.10-3.40 [m, 6H, $C\mathbf{H_2}N(C\mathbf{H_2})_2$], 3.90 (s, 3H, OCH_3), 6.99-8.20 (m, 12H, ArH), 15.78 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 1605 (C=O), 1350, 1170 (SO_2). Anal. Calcd



Scheme 1. Synthesis of new oxicam derivative series 8 and series 9.

for $C_{30}H_{30}F_3N_3O_5S$ (601.64): C, 59.89; H, 5.03; N, 6.98; Found: C, 60.25; H, 5.21; N, 6.88.

3-(4-methoxybenzoyl)-4-hydroxy-2-{3-[4-(2-fluorophenyl)-1-piperazinyl]propyl}-2H-1,2-benzothiazine 1,1-dioxide **8f** 45% yield, mp 58–60°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.23 (brs, 2H, $CH_2C\mathbf{H_2}CH_2$), 2.01 (brs, 2H, $CH_2CH_2C\mathbf{H_2}$-$N_{piperazine}$), 2.29 (brs, 4H, $H_{piperazine}$), 2.95 [brs, 6H, $C\mathbf{H_2}N(C\mathbf{H_2})_2$], 3.90 (s, 3H, OCH_3), 6.88-8.20 (m, 12H, ArH), 15.71 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 1606 (C=O), 1352, 1176 (SO_2). Anal. Calcd for $C_{29}H_{30}FN_3O_5S$ (551.63): C, 63.14; H, 5.48; N, 7.62; Found: C, 62.79; H, 5.86; N, 7.27.

4-hydroxy-3-(4-methylbenzoyl)-2-{3-[4-(2-pyrimidyl)-1-piperazinyl]propyl}-2H-1,2-benzothiazine 1,1-dioxide **8g** 19% yield, mp 135–138°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.26 (brs, 2H, $CH_2C\mathbf{H_2}CH_2$), 1.95-2.12 [m, 6H, $C\mathbf{H_2}N(C\mathbf{H_2})_2$], 2.44 (s, 3H, CH_3), 3.20 (brs, 2H, $C\mathbf{H_2}CH_2CH_2$), 3.66 (brs, 4H, $H_{piperazine}$), 6.47 [brs, 1H, $CH(5)_{pyrimidine}$], 7.30-8.21 (m, 8H, ArH), 8.27-8.29 [d, J = 6 Hz, 2H, $CH(4\text{ and }6)_{pyrimidine}$], 15.70 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 1600 (C=O), 1349, 1180 (SO_2). Anal. Calcd for $C_{27}H_{29}N_5O_4S$ (519.62): C, 62.41; H, 5.63; N, 13.48; Found: C, 62.11; H, 5.80; N, 13.67.

3-(4-fluorobenzoyl)-4-hydroxy-2-{3-[4-(3-trifluoromethyl-phenyl)-1-piperazinyl]propyl}-2H-1,2-benzothiazine 1,1-dioxide **8i** 48% yield, mp 140–143°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.29 (brs, 2H, $CH_2C\mathbf{H_2}CH_2$), 2.01(brs, 2H, $CH_2CH_2C\mathbf{H_2}N_{piperazine}$), 2.29 (brs, 4H, $H_{piperazine}$), 3.10 [brs, 6H, $C\mathbf{H_2}N(C\mathbf{H_2})_2$], 6.98-8.22 (m, 12H, ArH), 15.51 (s, 1H, OH_{enolic}). FT-IR (cm^{-1}): 1605 (C=O), 1355, 1175 (SO_2). Anal. Calcd for $C_{29}H_{27}F_4N_3O_4S$ (589.60): C, 59.08; H, 4.62; N, 7.13; Found: C, 59.40; H, 4.82; N, 7.02.

Synthesis of *2-[2-(4-arylpiperazinyl)-2-oxoethyl]-4-hydroxy-3-(4-substituted-benzoyl)-2H-1,2-benzothiazine 1,1-dioxides* **9a-g**

5 mmol of corresponding 1,2-benzothiazine **4a-d** were dissolved in 20 ml of anhydrous EtOH with addition of 5 ml of EtONa (0.115 g of Na in 5 ml of anhydrous

ethanol). Then, 5 mmol of corresponding 1-(2-chloro-1-oxoethyl)-4-arylpiperazine **6a–c** or 2-(2-chloro-1-oxoethyl)-1,3,4,9-tetrahydro-*β*-carboline **7** were added and refluxed with stirring for about 10 h. When the reaction ended, which was monitored on TLC plates, ethanol was distilled off, the residue was treated with 50 ml of $CHCl_3$ and insoluble materials were filtered off. The filtrate was then evaporated and the residue was purified by crystallization from ethanol to give desirable products **9a-g**. Compounds **9f** and **9g** were previously described (Szczęśniak-Sięga *et al.*, 2014).

3-benzoyl-4-hydroxy-2-[2-(1,3,4,9-tetrahydro-β-carbolin-2-yl)-2-oxoethyl]-2H-1,2-benzothiazine 1,1-dioxide **9a** 30% yield, mp 215–217ºC; ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 3.33 [m, 4H, C**H**$_2$(3 and 4)$_{carboline}$], 4.16-4.29 [m, 4H, C**H**$_2$CO andC**H**$_2$(1)$_{carboline}$], 6.91-8.15 (m, 13H, ArH), 10.69-10.72 (m, 1H, NH), 15.04 (s, 1H, O**H**$_{enolic}$). FT-IR (cm^{-1}): 3324 (NH), 1642, 1620 (C=O), 1347, 1180 (SO_2). Anal. Calcd for $C_{28}H_{23}N_3O_5S$ (513.56): C, 65.48; H, 4.51; N, 8.18; Found: C, 65.25; H, 4.44; N, 8.05.

3-benzoyl-4-hydroxy-2-{2-[4-(2-pyrimidyl)-1-piperazinyl]-2-oxoethyl}-2H-1,2-benzothiazine 1,1-dioxide **9b** 40% yield, mp 168–169ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.13-4.22 (m, 10H, C**H**$_2$CO and 8H$_{piperazine}$), 6.50-8.29 (m, 12H, ArH), 15.60 (s, 1H, O**H**$_{enolic}$). FT-IR (cm^{-1}): 1670, 1590 (C=O), 1340, 1175 (SO_2); Anal. Calcd for $C_{25}H_{23}N_5O_5S$ (505.54): C, 59.39; H, 4.59; N, 13.85; Found: C, 59.21; H, 4.45; N, 13.76.

3-benzoyl-4-hydroxy-2-{2-[4-(2-fluorophenyl)-1-piperazinyl]-2-oxoethyl}-2H-1,2-benzothiazine 1,1-dioxide **9c** 56% yield, mp 140–145ºC; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* (ppm): 1.66 (brs, 2H, C**H**$_2$CO), 2.86-3.55 (m, 8H, H$_{piperazine}$), 6.78-8.27 (m, 13H, ArH), 15.60 (s, 1H, O**H**$_{enolic}$). FT-IR (cm^{-1}): 1732, 1672 (C=O), 1344, 1180 (SO_2); Anal. Calcd for $C_{27}H_{24}FN_3O_5S$ (521.56): C, 62.18; H, 4.64; N, 8.06; Found: C, 62.30; H, 4.78; N, 7.84.

4-hydroxy-3-(4-methylbenzoyl)-2-{2-[4-(2-pyrimidyl)-1-piperazinyl]-2-oxoethyl}-2H-1,2-benzothiazine 1,1-dioxide **9d** 41% yield, mp 205–207ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.43 (s, 3H, CH_3), 3.15-4.21 (m, 10H, C**H**$_2$CO and 8H$_{piperazine}$), 6.50-8.29 (m, 11H, ArH), 15.65 (s, 1H, O**H**$_{enolic}$). FT-IR (cm^{-1}): 1665, 1600 (C=O), 1340, 1180 (SO_2); Anal. Calcd for $C_{26}H_{25}N_5O_5S$ (519.57): C, 60.10; H, 4.85; N, 13.48; Found: C, 60.15; H, 4.86; N, 13.41.

3-(4-fluorobenzoyl)-4-hydroxy-2-{2-[4-(3-trifluoromethylphenyl)-1-piperazinyl]-2-oxoethyl}- 2H-1,2-benzothiazine 1,1-dioxide **9e** 54% yield, mp 158–160ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.05-3.32 (m, 10H, C**H**$_2$CO and 8H$_{piperazine}$), 6.99-8.27 (m, 12H, ArH), 15.49 (s, 1H, O**H**$_{enolic}$). FT-IR (cm^{-1}): 1666, 1610 (C=O), 1345, 1180 (SO_2); Anal. Calcd for $C_{28}H_{23}F_4N_3O_5S$ (589.56): C, 57.04; H, 3.93; N, 7.13; Found: C, 57.37; H, 3.80; N, 6.95.

Cells

Normal human dermal fibroblasts (NHDF) from adult donors, were purchased from Lonza (Verviers, Belgium). Normal cell line V79 (fibroblasts from Chinese Hamster lung) and human cancer cell lines: A549 (pulmonary basal cell alveolar adenocarcinoma), LoVo (colon adenocarcinoma) were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

Cell and culture conditions

Cells were grown in the culture media recommended by the cell line supplier. Before the test, adherent cells were detached with the trypsin EDTA solution, and FBS containing medium was used to neutralize the effects of Trypsin/EDTA solution. Then, the cells were spun down, counted, stained with a 0.4% solution of trypan blue, and inspected under a microscope for cell viability. Afterwards, cells were placed in 96-well plastic culture plates (2×10^3 cells/well) and incubated at 37°C in a CO_2-incubator for 24 h. Next, the cells were incubated with different concentrations of all of the tested compounds (5, 10, 20, 50 and 100 μM). The cultures were incubated for 48 h in the CO_2-incubator at 37°C, after which the cells were harvested and used for cell proliferation test.

Determination of cell proliferation

Cell proliferation was estimated with the sulforhodamine B (SRB)-colorimetric assay (Vichai *et al.,* 2006). Briefly, cell cultures were fixed with cold TCA (final concentration 10% w/v) in cultures of adherent cells for 1 h at 4°C, then washed four times with tap water and air-dried at room temperature (20–25°C). A mildly acidic SRB solution (0.4% dye solution in 1% acetic acid) was added to each well for 30 min at 25°C, and then unbound stain was removed by rinsing with an aqueous solution of 1% (v/v) acetic acid. Culture plates were then allowed to dry at room temperature. The protein-bound dye was dissolved in 10 mM Tris base solution (pH 10.5) for 10 min on a gyratory shaker. Absorbance of the SRB solution was estimated at 540 nm in a *Victor 2* microplate reader (Perkin-Elmer, MA, USA).

Evaluation of Intracellular ROS Level

Intracellular ROS levels were determined using non-polar, non-fluorescent 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a marker of oxidative stress at final concentration of 25 μM. Following treatment with all of the tested compounds for 4 h, the V79 cell cultures were incubated with a medium (200 μl) with non-fluorescence probe DCFH-DA for the last 2 h of culture in the dark at 37°C and 5% CO_2, according to a standard procedure (Eruslanov *et al.*, 2010). Then, the cells were washed twice with PBS and treated with H_2O_2 (100 μM) for 30 min. DCFH-DA probe penetrates through the membrane by passive diffusion and then it is hydrolyzed enzymatically by intracellular esterases to the polar, non-fluorescent form of 2',7'–dichlorodihydrofluorescin (DCFH). DCFH is immediately oxidized in the presence of reactive oxygen species (ROS) to highly fluorescent 2',7'-dichlorofluorescin (DCF).

Fluorescence of DCF was measured (λ_{ex}=485 nm, λ_{em}=535 nm) in a Victor 2 microspectrophotometer (PerkinElmer, Waltham, MA, USA). The applied concentration of H_2O_2 was 100 μM. Thirty-minute cell incubation with H_2O_2 was chosen, as data found in the literature strongly suggest that cells were able to decompose almost all of the H_2O_2 added to the culture medium within 30 min (Loo *et al.*, 2012). The test results are presented as the E/E_0 ratio. The E/E_0 ratio is the comparison of the test sample value (E) to the control value (E_0). The effect of increased generation of ROS was observed in the H_2O_2 induced cells in contrast to the non-oxidized stress cells. H_2O_2 is extensively used to induce oxidative stress in vitro. The products of H_2O_2, i.e. superoxide and hydroxyl radicals, are major components of ROS.

In vitro cyclooxygenase inhibitory activity

COX Colorimetric Inhibitor Screening Assay Kit (Cayman Chemical Company, Ann Arbor, MI). This method allows to estimate the peroxidase activity

of COX by colorimetric monitoring of occurrence of the oxidized form of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm. TMPD is a substrate for most enzymes with peroxidase activity, and high throughput microplate assays using TMPD allow the rapid screening of a wide range of therapeutics that inhibit COX activity *in vitro*. The test is based on oxidation of TMPD during reduction of PGG_2 (prostaglandin G_2) to PGH_2, which is reflected by a change in color, measured spectrophotometrically (Victor 2 microspectrophotometer, PerkinElmer Waltham, MA, USA). The assay uses Tris-HCl buffer (0.1 M assay buffer, pH 8.0), a solution of heme in dimethylsulfoxide (DMSO), enzymes (COX-1, COX-2), arachidonic acid (100 μM), KOH (0.1 M) and a solution of TMPD. The assay mixture contains: 150 μL of assay buffer, 10 μL of heme, and 10 μL of COX-1 or COX-2. To determine 100% enzyme activity (each COX sample was assayed in triplicate) 10 μL of the substances used as solvents (methanol, ethanol, DMSO) were added to the wells. 10 μL of the tested inhibitors at appropriate concentrations were added to the other wells. 20 μL of TMPD were added to all of the wells. The reaction was initiated by the addition of arachidonic acid. The effect of the inhibitors tested on COX-1 and COX-2 enzyme activity was measured by assaying the rate of TMPD oxidation within 2 min in a spectrophotometer at 590 nm. We determined the activity factor at 2 min of incubation with the tested compounds in comparison to the initial activity of the enzyme. This enabled the calculation of IC_{50} values (concentrations at which 50% inhibition of the enzyme's activity occurred).

Statistical analysis

Statistical significance of the results was calculated with the paired t-test and with Two-way analysis of variance ANOVA, following routine statistical methods.

RESULTS AND DISCUSSION

Chemistry

The synthesis of the novel oxicam derivatives was performed according to the reaction pathways illustrated in Scheme 1. Compounds **3a-d** and **4a-d** were synthesized according to the described procedures (Krzyżak *et al*., 2014; Szczęśniak-Sięga *et al*., 2014; Yoshimura *et al*., 2015). Briefly, commercially available saccharine **1** was condensed with appropriate bromoacetophenones **2a-d** in the presence of dimethylformamide (DMF) and triethylamine (TEA) at room temperature. Then, the obtained **2a-d** compounds were converted by Gabriel-Colman rearrangement into compounds **3a-d**. Compounds **5a-d** and **6a-c** were also synthesized according to the previously described procedure (Lopez *et al*., 2010; Krzyżak *et al*., 2014; Maniewska *et al*., 2014; Muszalska *et al*., 2015; Paudel *et al*., 2016), by alkylation or acylation of the corresponding arylpiperazines. Whereas compound **7** was obtained in a reaction of chloroacetyl chloride with 2,3,4,9-tetrahydro-1H-β-carboline (Zhou *et al*., 2016).

The final compounds were obtained by **4a-d** alkylation with **5a-d**, **6a-c** or **7** in the presence of sodium ethoxide (EtONa) with medium yields. All new compounds were divided into two series – **series 8** with a propylene linker between thiazine and piperazine nitrogens, and **series 9** with an 2-oxoethylene linker in the same position.

Table 1. IC_{50} values (SD) calculated for COX-1 and COX-2 activities at 2 min of incubation with the tested compounds (mean S.D.; n=3)

Compound	IC_{50} [μM], (SD)		Ratio: COX-2/COX-1
	COX-1	COX-2	
8a	NA	NA	
8b	69.3 (11.4)*	NA	
8c	NA	NA	
8d	NA	NA	
8e	NA	NA	
8f	NA	NA	
8g	NA	NA	
8h	NA	NA	
8i	NA	NA	
9a	NA	NA	
9b	63.0 (3.9)*Δ	924.2 (268.8)*Δ	14.7
9c	157.1 (35.3)Δ	NA	
9d	NA	NA	
9e	NA	NA	
9f	NA	NA	
9g	NA	NA	
meloxicam	85.8 (12.5)	71.5 (5.1)	0.8
piroxicam	170.5 (15.7)	127.6 (11.9)	0.7

The *t*-test paired to evaluate the inhibition of COX-1 and COX-2 activity compared to the piroxicam control compound (*p<0.05) and the meloxicam control compound (Δp<0.05).

In vitro cyclooxygenase inhibitory activity

The impact of piroxicam and meloxicam (the standard inhibitors of cyclooxygenases from the group of oxicams) on COX-1 and COX-2 activities at 2 min of incubation, as recommended for the test kit carried out in comparison to 16 studied compounds, was tested. The IC_{50} values (i.e. the concentration of the tested compounds in μM which can exert 50% inhibition of the enzyme's activity) were calculated separately for COX-1 and COX-2 activity estimations at 2 min of incubation with the tested compounds. Selectivity of the compounds for COX-1 or COX-2 was assessed by calculation of the IC_{50} ratios. The IC_{50} values were not calculated for those tested compounds which exerted very low or no inhibitory activity at 2 min of incubation. Results of the calculations are given in Table 1.

Compounds **8a**, **8c-8i**, **9a** and **9d-9g** showed no *in vitro* COX inhibitory activity when tested with the method applied. Whereas, compounds **8b**, **9b** and **9c** exerted significant COX-1 selectivity at lower doses than piroxicam. Compounds **8b** and **9b**, which possess a pyrimidine ring in their structure and an unsubstituted benzene ring (R=H) at the thiazine 3 position, have even shown a greater COX-1 inhibition than the meloxicam standard (IC_{50}=69.3 and 63.0 *vs* IC_{50}=85.8, respectively). Compound **9c** also possesses an unsubstituted benzene

Table 2. ROS scavenging activity of the tested compounds (mean S.D.; n 3); the results were compared to the relative control samples (without tested compounds; E0) and expressed as E/E0 ratios

	without H_2O_2			with H_2O_2		
compound	mean E/E_0	S.D.	*p*	mean E/E_0	S.D.	*p*
8a	0.95	0.02	NS	1.05	0.01	0.02*
8b	0.99	0.04	NS	1.10	0.25	NS
8c	0.88	0.03	NS	0.84	0.01	0.001*
8d	0.85	0.01	NS	0.87	0.05	NS
8e	0.92	0.07	NS	0.90	0.03	0.04*
8f	0.89	0.01	0.001*	0.74	0.14	NS
8g	1.05	0.04	NS	1.04	0.17	NS
8h	0.94	0.06	NS	0.87	0.14	NS
8i	0.81	0.06	0.04*	0.86	0.15	NS
9a	0.89	0.05	NS	1.05	0.01	0.03*
9b	1.03	0.04	NS	1.11	0.00	NS
9c	0.92	0.06	NS	0.88	0.02	0.01*
9d	0.95	0.17	NS	1.15	0.22	NS
9e	0.84	0.03	0.01*	0.72	0.12	NS
9f	1.14	0.03	0.02*	1.35	0.04	0.01*
9g	1.00	0.04	NS	1.14	0.18	NS
meloxicam	0.99	0.20	NS	1.22	0.14	NS
piroxicam	1.19	0.17	NS	1.59	0.25	NS
ascorbic acid	0.80	0.12	NS	0.18	0.07	0.001*
trolox	0.78	0.08	0.01*	0.15	0.05	0.001*

*Statistical significance (*p*) was assessed with the *t*-test paired ($p<0.05$)

ring (R=H) at the thiazine 3 position, but instead of the pyrimidine ring it has an o-fluorophenyl substituent and shows less inhibition of COX-1 than meloxicam, but stronger than piroxicam (IC_{50}=170.5>157.1>85.8, respectively). Only compound **9b** exhibited COX-2 inhibition as well, but that was at higher dose then the reference drugs – piroxicam and meloxicam (IC_{50}=924.2 > 127.6>71.5, respectively). The structure-activity relationship (SAR) in this group of compounds is rather difficult to determine due to the fact that only 3 out of 16 tested compounds showed COX-1/COX-2 inhibitory activity.

Evaluation of Intracellular ROS Level

The ROS scavenging activity of 16 new compounds and two oxicams - piroxicam and meloxicam – in the presence and absence of oxidative stress (generated by addition of H_2O_2 to cell cultures) is shown in Table 2. Compounds **8f**, **8i** and **9e** showed significant ROS scavenging activity under normal conditions. In contrast, compound **9f** caused an increase in the ROS concentration under normal conditions, as well as during oxidative stress (induced by adding H_2O_2). However, compounds **8a**, **8c**, **8e**, **9a** and **9c** were very efficient at ROS scavenging under oxidative stress, in contrast to oxicams. Moreover, there seems to be no structure–activity relationship between the structure of the new compounds and their ROS scavenging activity measured by the method applied. The antioxidant activity of all of the tested compounds was lower than standard ROS scavengers – ascorbic acid and trolox.

Determination of cell proliferation

Cell proliferation was estimated with the sulforhodamine B (SRB)-colorimetric assay (Vichai *et al.*, 2006). New compounds and two reference drugs (piroxicam and meloxicam) were tested on two normal cell lines (normal human dermal fibroblasts NHDF and fibroblast from Chinese Hamster lung V79) and two human cancer cell lines (colon adenocarcinoma, LoVo, and pulmonary basal cell alveolar adenocarcinoma, A549). The results are shown in Table 3. All compounds from the **series 8** did not inhibit the growth of both, normal and cancerous cells. On the other hand, compounds from **series 9** displayed a significant activity in inhibiting the growth of cancer cells (both, LoVo and A549, or only one of them), with the exception of compound **9e**. Compound **9c** showed the highest therapeutic index (the largest difference between concentrations inhibiting 50% growth of normal and cancerous cells), among the tested compounds. Therefore, the presence of a two-carbon aliphatic linker with a carbonyl group between the thiazine and piperazine nitrogens in **series 9**, instead of a three-car-

Table 3. IC_{50} values (S.D.) calculated for NHDF, V79, LoVo and V79 cell lines at 48 h of incubation with the tested compounds (mean S.D.; n = 3)

	IC_{50} [μM], (S.D.)			
compound	*NHDF*	V79	LoVo	A549
8a	NA	NA	NA	NA
8b	NA	NA	NA	NA
8c	NA	NA	NA	NA
8d	NA	NA	NA	NA
8e	NA	NA	NA	NA
8f	NA	NA	NA	NA
8g	NA	NA	NA	NA
8h	NA	NA	NA	NA
8i	NA	NA	NA	NA
9a	NA	199.42 (43.0)	NA	411.1 (46.8)
9b	131.3 (20.4)	128.56 (7.9)	126.6 (7.9)	163.8 (2.8)
9c	433.3 (184.5)	247.17 (37.5)	243.9 (55.7)	347.3 (46.1)
9d	259.0 (107.8)	207.2 (80.2)	504.5 (316.0)	542.8 (181.2)
9e	474.5 (291.6)	282.0 (169.1)	NA	NA
9f	136.5 (17.6)	129.3 (34.9)	124.2 (7.1)	185.9 (9.0)
9g	283.3 (123.5)	182.8 (80.4)	NA	437.5 (191.0)
meloxicam	205.6 (44.2)	231.8 (33.5)	124.6 (11.2)	148.3 (37.9)
piroxicam	170.5 (23.0)	200.0 (32.9)	122.1 (9.6)	138.1 (27.8)

bon linker in **series 8**, seems to be crucial for cytotoxic activity of the compounds studied. The cytotoxic activity of the new compounds against the LoVo line seems to be particularly important, as studies in recent years have proved that NSAIDs are very useful in colorectal cancer chemoprevention (Wakeman *et al.*, 2017).

CONCLUSIONS

The COX inhibiting activity, the ROS scavenging activity, and cytotoxicity of the 16 new oxicam derivatives were examined in order to assess the chemopreventive potential of the compounds studied. We have shown that COX inhibiting activity and cytotoxicity depend on the structure of the individual compounds. From the structure-activity analysis (SAR), it follows that the presence of a two-carbon aliphatic linker with a carbonyl group between the thiazine and piperazine nitrogens in **series 9**, instead of a three-carbon (propylene) linker in **series 8**, seems to be crucial for cytotoxic activity of the compounds studied (Table 3). Moreover, a fluorine substituent in the benzene ring of phenyl-piperazine moiety (compound **9c**) seems to be beneficial for COX-1 inhibition activity and cancerous cells cytotoxicity. Instead, there seems to be no structure–activity relationship between structure of the new compounds and their ROS scavenging activity tested by the method applied.

However, compound **9c** showed the highest therapeutic index, i.e. the largest difference between concentrations inhibiting 50% growth of normal and cancerous cells, among the compounds tested. Moreover, compound **9c** also displayed a significant statistical inhibition of COX-1which may cause the inhibition of platelet activation, facilitation of immunosurveillance and prevention of haematogenous spread of malignancy, and has been suggested as another putative mechanism of cancer prevention (Thun *et al.*, 2012). Additionally, compound **9c** was very efficient at ROS scavenging under oxidative stress, which is the next important factor in the chemoprevention of cancer (Kundu *et al.*, 2008). Such enzymes as cyclooxygenases (COX), cytochrome p450 enzymes and lipoxygenases, among others, are intercellular sources of ROS. These enzymes produce ROS as part of their normal enzymatic action and contribute to the intercellular source of ROS (Finkel, 2011). Therefore, increased COX-2 activity occurring in malignancies and inflammation, causes increased ROS levels and stimulates cancer development. Antioxidants may act by preserving normal cell cycle regulation, inhibition of proliferation and inducing apoptosis, inhibition of tumor invasion and angiogenesis, suppression of inflammation, and stimulation of phase II detoxification enzyme activity (Sen *et al.*, 2011).

The cytotoxic activity of the new compound **9c** against the LoVo cell line (colon adenocarcinoma) seems to be particularly important, as studies of recent years have proved that NSAIDs are very useful in colorectal cancer prevention (Tsioulias *et al.*, 2015). Due to the occurrence of multidrug resistance (MDR) of colorectal cancer, further studies are needed to investigate possible capabilities of compound **9c** to reverse this phenomenon.

Conflicts of interests

The authors hereby declare that there are no conflicts of interests.

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