The effects of trifluoromethylated derivatives on prostaglandin E_2 and thromboxane A_2 production in human leukemic U937 macrophages

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ABSTRACT

Background: Convenient approach to modulation of the inflammation is influence on production of inflammatory mediators – eicosanoids, generated in arachidonic acid (AA) metabolism. Common therapeutic activity of non-steroidal anti-inflammatory drugs (NSAID), such as aspirin, includes inhibition of two crucial enzymes of AA metabolism - cyclooxygenase-1 and -2 (COX-1/2), with certain risk for gastrointestinal and renal intolerance. Ever since enrolment of COX-2, particularly overabundance of its main products prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2) in numerous pathological processes was recognized, it became significant therapeutic target.

Objective: The aim of this study was to examine effects of synthesized organo-fluorine compounds on PGE_2 and TXA_2 production in inflammation process.

Methods: Trifluoromethyl compounds were synthesized from *N*-benzyl trifluoromethyl aldimine, commercially available 2-methyl or 2-phenyl α-bromo esters (β-lactams *trans*-**1** and *trans*-**2** and trifluoromethyl β-amino ester, respectively) and methyl 2-isocyanoacetate (2-imidazoline *trans*-**4**). The reactions proceeded with high geometric selectivity, furnishing the desired products in good yields. The influence of newly synthesized compounds on PGE_2 and TXA_2 production in human leukemic U937 macrophages on both enzyme activity and gene expression levels was observed.

Results: Among tested trifluoromethyl compounds, methyl *trans*-1-benzyl-5-(trifluoromethyl)-4,5-dihydro-1*H*-imidazole-4-carboxylate (*trans*-4) can be distinguished as the most powerful anti-inflammatory agent, probably due to its trifluoromethyl-imidazoline moiety.

Conclusions: Some further structural modification of tested compounds and particularly synthesis of different trifluoromethyl imidazolines could contribute to development of new COX-2 inhibitors and potent anti-inflammatory agents.

Keywords: inflammation, trifluoromethyl derivatives, cyclooxygenase, macrophage.

1. INTRODUCTION

Convenient approach to modulation of the inflammation is influence on production of inflammatory mediators – eicosanoids, generated in arachidonic acid metabolism. Upon activation of enzyme phospholipase A_2 (PLA_2) by different inflammatory stimuli, arachidonic acid (AA) is released from membrane phospholipids and converted to structurally diverse eicosanoids, by three classes of enzymes: cyclooxygenases (COX), lipoxygenases (LOX) and epoxygenases. COX pathway mainly implies activities of two isoenzymes, COX-1 and COX-2, followed by prostaglandin and thromboxane synthases (PGES and TXAS, respectively) and resulting in formation of prostaglandins (PGs), such as PGE_2, PGI_2 (prostacyclin), PGD_2, $PGF_{2\alpha}$, as well as thromboxane A_2 (TXA_2). COX-1 and COX-2 are structurally similar, but they are encoded by distinct genes and have distinct functions: COX-1 is constitutively expressed in virtually all cells, and is responsible for platelet aggregation, gastric cytoprotection and renal water balance, while COX-2 is highly up regulated in macrophages, monocytes, fibroblasts and endothelial cells during inflammation [1, 2]. Regarding products of interest in our research, PGE_2 is the most abundant prostanoid in the human body, with a variety of bioactivities (blood pressure regulation, protection of gastrointestinal mucosa, ovulation, renal function), but also implicated in promotion of fever, pain, chronic inflammation and related diseases: atherosclerosis, arthritis, allergy, inflammatory bowel disease, diabetes, neurodegeneration, cancer, etc. [3, 4]. Furthermore, TXA_2 is a vasoconstrictor, and its known as a powerful platelet stimulator and inducer of platelet aggregation. It has a half life of 30 s under physiological conditions and is converted to the stable, biologically inactive metabolite TXB_2 [5].

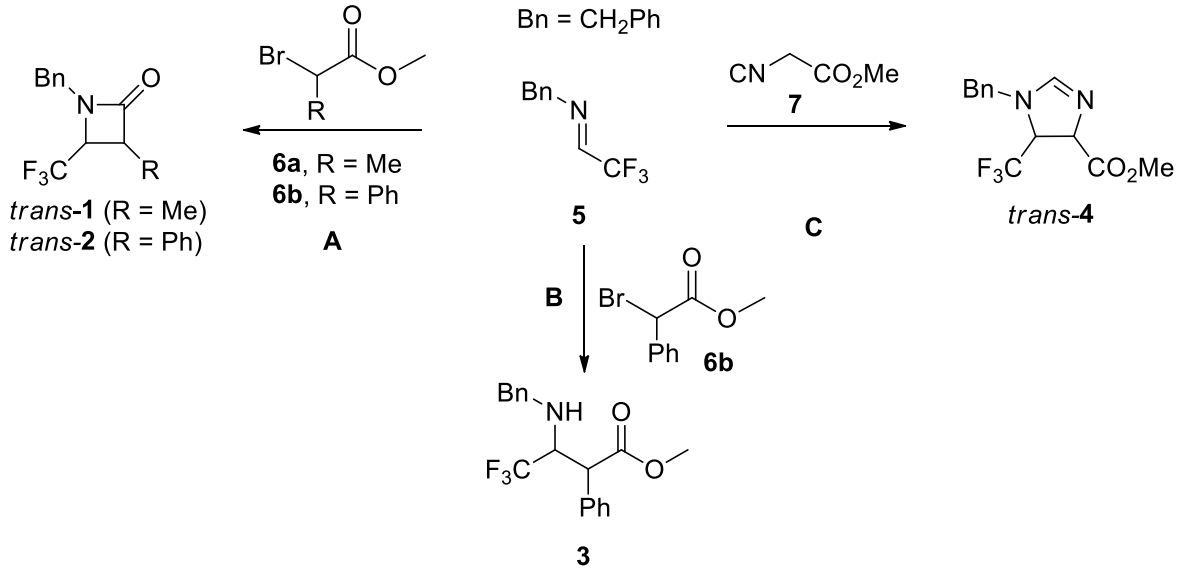
Organo-fluorine compounds occupy a unique place in organic chemistry, fluorine being the 13th most abundant element in the Earth's crust. Organo-fluorine compounds often have been used as pharmaceutical [6-9] and agrochemical materials [10-12]. Indeed, it is estimated that approximately 20% of modern pharmaceuticals and 30% of agrochemicals contain fluorine [13]. Very recently the research groups of Ye [14] and Kirschning [15] reported the anti-inflammatory activity of aromatic and heteroaromatic trifluoromethyl derivatives, respectively. Taking into account the medical applications of β-lactams [16] and imidazolines [17], present paper is aimed at evaluating potential anti-inflammatory activity of related trifluoromethyl derivatives through the effects on prostaglandin E_2 and tromboxane A_2 production.

2. MATERIALS AND METHODS

2.1. Chemistry

2.1.1. Synthesis of trifluoromethyl compounds

1-Benzyl-3-methyl-4-(trifluoromethyl)azetidin-2-one (*trans*-**1**), and 1-benzyl-3-phenyl-4-(trifluoromethyl)azetidin-2-one (*trans*-**2**) were chemoselectively synthesized following a recently reported procedure [18], involving heterogeneous Reformatsky reaction conditions (activated Zn dust/THF, 40 °C, 18 h), starting from *N*-benzyl trifluoromethyl aldimine **5** [19] and commercially available methyl 2-bromo esters **6a**,**b** (Scheme 1, **A**). Fluorinated β-lactams *trans*-**1** and *trans*-**2** were purified by flash chromatography on silica gel.



Scheme 1

Starting from methyl bromo(phenyl)acetate **6b**, a modified Reformatsky reaction [20, 21] was successfully performed under Barbier conditions (activated Zn dust/DMF, r.t., 3 h), affording the expected methyl 3-(benzylamino)-4,4,4-trifluoro-2-phenylbutanoate (**3**) in satisfactory yields (Scheme 1, **B**). Finally, methyl *trans*-1-benzyl-5-(trifluoromethyl)-4,5-dihydro-1*H*-imidazole-4-carboxylate (*trans*-**4**) was synthesized by an Ag_2O-catalyzed Mannich-type reaction [22] even starting from trifluoromethyl aldimine **5** and commercially available methyl 2-isocyanoacetate **7** (Scheme 1, **C**). After reaction work-up, the desired *trans*-**4** was purified by flash chromatography on silica gel.

2.1.2. 1-Benzyl-3-methyl-4-(trifluoromethyl)azetidin-2-one (*trans*-1), 1-Benzyl-3-phenyl-4-(trifluoromethyl)azetidin-2-one (*trans*-2), Methyl 3-(benzylamino)-4,4,4-trifluoro-2-

phenylbutanoate (3), Methyl *trans*-1-benzyl-5-(trifluoromethyl)-4,5-dihydro-1*H*-imidazole-4-carboxylate (*trans*-4)

Synthesis and spectroscopic characterization of tested compounds are available in the following publications: *trans*-**1** and *trans*-**2** in [18]; **3** in [20]; and *trans*-**4** in [22].

2.2. Biological activity

2.2.1. Cell culture and transformation protocol

U937 cells (human leukemic myelomonocytic cell line) were maintained in suspension culture in RPMI-1640, supplemented with 10% (v/v) fetal bovine serum (FBS), 2.05 mM L-glutamine, 25 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B, at 37 °C, in a humidified atmosphere of 5% CO_2. The density of cells was kept between 2×10^5 and 2×10^6 cells/mL.

For differentiation into macrophages, 2×10^6 monocytes were seeded in 6-well plates containing 2 mL of complete culture medium and 100 nM phorbol 12-myristate 13-acetate (PMA). After 72 hours, supernatant was removed and fresh, serum-free medium was added.

2.2.2. Cytotoxicity

Monocytes (1×10^6 cells/mL) were cultured in serum-free medium containing tested compounds, solvent or equal amount of medium (control) for 22 hours. Morphological changes in the cells were observed microscopically, and the survival rates were evaluated by trypan blue exclusion 22 hours after treatments.

2.2.3. PGE_2 and TXA_2 release from U937 macrophages

Analysis of PGE_2 and TXA_2 release in U937 cells is based on modified methods of Penglis [5] and Wong [23]. In brief, macrophages (2×10^6 cells, 2 mL of serum-free medium) were pre-treated with tested compounds (*trans*-**1**, *trans*-**2**, **3** and *trans*-**4** in sub-toxic concentrations or solvent - DMSO) for 2 hours and stimulated with 0.5 μg/mL LPS for 20 hours. Afterward, 1 μL of 20 mM arachidonic acid was added and cells were incubated at 37 °C with 5% CO_2 for 10 min. Acidification with cold 1% aqueous formic acid (0.4 mL) to pH 3 terminated the reaction and internal standard, prostaglandin B_2 (10 μL of 30 μg/mL solution in medium) was added. Extraction of products and internal standard was done according to [24].

TXA_2 has a half life of 30 s under physiological conditions and is converted to the stable metabolite TXB_2, so the levels of PGE_2, TXB_2 and internal standard PGB_2 were determined by LC-MS/MS, according to previously published procedure [24, 25].

2.2.4. Gene expression levels in U937 macrophages by Real–Time Polymerase Chain Reaction (RT–PCR)

Macrophages (2×10^6 cells, 2 mL of serum-free medium) maintained in 6-well plates were pre-treated with tested compounds (*trans*-**1**, *trans*-**2**, **3** and *trans*-**4** in sub-toxic concentrations or solvent - DMSO for control) for 2 hours and stimulated with 0.5 μg/mL LPS for additional 20 hours (Jiang et al., 2003). All samples and control were made in triplicate. Expression of PLA_2, COX-1 and COX-2 genes (mRNA) was determined.

RNA extraction was carried out using TRIzol®/chloroform extraction and isopropyl alcohol precipitation [26]. The concentration of extracted RNA was measured using Qubit RNA BR Assay Kit, on Qubit 2.0 Fluorometer (Invitrogen by Life Technologies), as described by the manufacturer`s procedure. After DNase I treatment (Ambion by Life Technologies), cDNA synthesis was performed using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems by Life Technologies). Real-time quantitative gene analysis was performed using Stratagene M×3005P Agilent Technologies and a Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences which were used are shown in **Table 1**. Gene expression was normalized to GAPDH and represented as ΔCt values. For each sample the mean of the ΔCt values was calculated. Relative gene expression was normalized to 1.0 (100%) of controls.

Table 1. Primer sequences used in this study

gene	Forward (5'→3')	Reverse (5'→3')
GAPDH (human)	TGG TAT CGT GGA AGG ACT C	AGT AGA GGC AGG GAT GAT G
COX-1 (human)	GAG CTC TGA GGG GCA GGA AA	AAC CCC AAC ACT CAC CAT GC
COX-2 (human)	CAG CAC TTC ACG CAT CAG TT	CGC AGT TTA CGC TGT CTA GC
PLA_2 (human)	CAT CTC GTT GCT CTG TTT CCC	TGC TGC CGT TTC CAG TAC AT

2.2.5. Statistical analysis

The experimental results were expressed as mean values ± SD of three different trials. The differences between the samples were evaluated using one-way analysis of variance (ANOVA) followed by comparison of the means by Tukey HSD test ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

Trifluoromethyl β-lactams *trans*-**1** and *trans*-**2** were synthesized under heterogeneous Reformatsky reaction conditions by using activated Zn dust, in THF, at 40 °C for 18 h. Under these conditions the β-amino ester intermediates undergo in *situ* a very fast intramolecular cyclization, leading to reported heterocyclic compounds as only reaction products. On the contrary, methyl 3-(benzylamino)-4,4,4-trifluoro-2-phenylbutanoate (**3**) was formed working under classical Barbier conditions, namely in the presence of activated Zn dust, but using DMF as solvent and stirring the mixture at r.t. for 3 h. Finally, 2-imidazoline *trans*-**4** was synthesized by an Ag_2O catalysed Mannich-type/cyclization cascade reaction between trifluoromethyl aldimine **5** and methyl 2-isocyanoacetate **7** in THF at room temperature. All the obtained compounds were analyzed by spectroscopic analysis (IR, ^{1}H, ^{13}C and ^{19}F NMR, HRMS) and the obtained data compared with those already reported in the literature, to confirm their structures.

The arachidonic acid metabolism is one of the major components of the inflammatory response, since arachidonic acid is immediately released from traumatized cellular membranes and converted to biologically active lipids which instantly act. Regarding cyclooxygenase pathway, PLA_2 activity is required to initiate arachidonic acid conversion, but strong up regulation of COX-2 in inflammation-related cell types is the key step in overexpression of prostaglandins, as well as thromboxane. When level of PGE_2 produced by induced COX-2 exceeds level of homeostatic, COX-1 mediated PGE_2, inflammation symptoms occur: pain, fewer, redness, or, concerning TXA_2, more intense vasoconstriction and platelet stimulation. It is well known that lasting, extensive COX-2 expression and activity are deeply involved in the pathogenesis of chronic inflammation and related diseases, such as atherosclerosis, arthritis, cancer etc. [1, 2, 27]. Ever since enrolment of COX-2 in numerous pathological processes was recognized, it became significant therapeutic target. Common therapeutic activity of non-steroidal anti-inflammatory drugs (NSAID), such as aspirin, includes both COX-1 and -2 inhibition, with certain risk for gastrointestinal and renal intolerance. Developed selective COX-2 inhibitors do not cause this side-effects, but are strongly implicated in cardiovascular complications [4, 28]. Therefore, there is certain need for finding novel compounds with potential to inhibit COX-2 enzyme activity, but also to obtain data on molecular mechanism of this action.

To test anti-inflammatory activity of compounds **1**, **2**, **3** and **4** a cell-based assay was used, with LPS as a standard COX-2 induction agent [5, 23]. To determine COX-2 inhibition potential, influence of newly synthesized compounds on PGE_2 and TXA_2 production in

human leukemic U937 macrophages on both enzyme activity and gene expression levels was observed. Macrophages were treated with sub-toxic concentrations of tested compounds (**Table 2**.), afterwards the amounts of produced PGE_2 and TXA_2 were determined by LC-MS/MS (**Table 3**.). Expression levels of PLA_2, COX-1 and COX-2 genes (mRNA) are shown in **Table 4**.

Table 2. Cytotoxicity of tested compounds

	Percent of viable cells (treated / control) (%)					
	Final concentration of tested compounds (μM)					Percent of viable cells in control
Compound	100	50	25	12.5	6.25	0.05% DMSO
***trans*-1**	89.8 ± 2.42	99.9±0.78	> 95%	> 95%	> 95%	> 95%
***trans*-2**	> 95%	> 95%	> 95%	> 95%	> 95%	> 95%
3	46.1 ± 2.53	85.8±3.95	> 95%	> 95%	> 95%	> 95%
***trans*-4**	> 95%	> 95%	> 95%	> 95%	> 95%	> 95%

Table 3. PGE_2 and TXA_2 release from U937 macrophages

	Product response ratios in probe / Product response ratios in control					
	***trans*-1** (50 μM)	***trans*-2** (50 μM)	**3** (25 μM)	***trans*-4** (50 μM)	Aspirin (25μM)	control
product						
TXA_2	0.65 ± 0.002 b	0.81 ± 0.03 b	0.55 ± 0.11 b	0.51 ± 0.01 b	0.31 ± 0.09 b	1.00 ± 0.01 a
PGE_2	0.83 ± 0.01 b	0.85 ± 0.02 a	0.98 ± 0.01 a	0.72 ± 0.01 b	0.68 ± 0.01 b	1.00 ± 0.06 a

Response ratios - metabolite peak area/internal standard peak area
Values are means ± SD of three repetitions. Means within each row with different letters (a, b) differ significantly from control ($p < 0.05$).

Table 4. Gene expression levels in U937 macrophages

	gene mRNA/GAPDH mRNA (a.u.)				
	***trans*-1** (50 μM)	***trans*-2** (50 μM)	**3** (25 μM)	***trans*-4** (50 μM)	control
gene					
COX-1	0.64 ± 0.02 a	0.85 ± 0.11 a	0.56 ± 0.10 b	0.71 ± 0.02 a	1 ± 0.19 a
COX-2	0.42 ± 0.11 b	0.56 ± 0.13 b	0.53 ± 0.03 b	0.31 ± 0.04 b	1 ± 0.15 a
PLA_2	0.68 ± 0.02 b	0.64 ± 0.20 a	0.75 ± 0.07 b	0.59 ± 0.05 b	1 ± 0.01 a

Values are means ± SD of three repetitions. Means within each row with different letters (a, b) differ significantly from control ($p < 0.05$).

Fluorinated compounds **1**-**4** (Scheme 1) were chosen since all they contain a trifluoromethyl group bonded to aliphatic residues, namely a β-lactam (*trans*-**1** and *trans*-**2**), a β-amino ester (**3**) and a 2-imidazoline ring (*trans*-**4**).

It was determined that *trans*-**1**, tested at final concentration of 50 μM, showed moderate inhibition of TXA_2 and PGE_2 production, but it has a great inhibition potential of COX-2 expression and also alters PLA_2. Compound **3,** tested at final concentration of 25 μM, since higher were cytotoxic, and compound *trans*-**2** (50 μM) were, in general, moderately active. Interestingly, only *trans*-**2** significantly reduced COX-1 mRNA levels. Among tested compounds, *trans*-**4** (50 μM) had the highest influence on LPS induced COX-2 and PLA_2 mRNA levels, as well as significant overall inhibition of TXA_2 and PGE_2 synthesis, although it was not statistically significant higher than activities of other two enantiomers, *trans*-**1** and *trans*-**2**. It can be assumed that common structural feature of tested compounds characterized by a trifluoromethyl group bonded to aliphatic residues can significantly contribute to expressed activity. Relevance of organo-fluorine compounds has been attributed to the improved pharmacological properties that fluorine may provide, such as enhanced thermal and metabolic stability, bioavailability, lipophilicity, and overall biological activity. As known, the substitution of hydrogen atoms with fluorine atoms often induces chemical and physical changes in the organic molecules, giving their particular biological properties.

Potency to inhibit arachidonic acid metabolism can be additional value to the medical application of β-lactams. They are traditionally used as antibiotics, but some recent developments in other fields describe them as LHRH antagonists, cholesterol-absorption inhibitors, and anticancer agents [16]. Unfluorinated β-lactams are also active as *N*-acylethanolamine acid amidase (NAAA) inhibitors [29].

Regarding imidazoline derivatives, unfluorinated and some fluoroaryl 2-imidazolines have a potential for therapeutic applications in inflammatory disease area [17].

Unfluorinated poly(β-amino ester) are reported to possess synergistic chemo-anti-inflammatory effects. According to our results among all tested derivatives compound *trans*-**4** can be distinguished as the most powerful anti-inflammatory agent, probably due to its trifluoromethyl-imidazoline moiety.

To the best of our knowledge, there are no examples in which the trifluoromethyl function associated with β-lactam or 2-imidazoline rings or β-amino esters were investigated for anti-inflammatory activities by the means of monitoring particular products of arachidonic acid metabolism. The results of our study indicate that some further structural modification of

tested compounds and particularly synthesis of different trifluoromethyl-imidazolines could contribute to development of new COX-2 inhibitors and potent anti-inflammatory agents.

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Conflict of Interest: All authors declare no conflict of interest.

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