Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes, present in most of the living organisms and encoded by seven evolutionarily unrelated gene families: the α-, β-, γ-, δ-, ε-, η-, and θ-CAs. The CAs catalyse the basic reaction of reversible hydration of carbon dioxide to bicarbonate and proton. This reaction is fundamental for biochemical processes in all living organisms. Invertebrates and lower organisms contain all the seven members of CA gene families and, in some cases, multiple gene families with in the same organism. In contrast, vertebrates and mammals contain only α-CAs. In humans, the CAs regulate a broad range of physiological processes, such as respiration, transport of carbon dioxide/bicarbonate between the lung and metabolising tissues, pH homeostasis, and electrolyte secretion in many tissues and organs. There are 15 CA isoforms in humans of which 12 are catalytically active. Three are inactive due to the lack of one or more of the three-histidine residues required for enzymatic activity. The human CA isoforms differ from one another in cellular localisation, distribution in organs, levels of expression in tissues and their kinetic properties. The human CA isoforms have high sequence and structural similarities, and therefore only subtle differences exist in their active sites.

In humans, increased expression of CA isoforms have been linked to retinal and cerebral oedema, glaucoma, epilepsy and altitude sickness. hCA IX, a transmembrane CA isoform, is overexpressed under hypoxic conditions in solid tumours and is involved in the progression of cancer. Many CA inhibitors have been used for treating various human diseases. Even if the inhibitors achieve desirable therapeutic effects, they may cause unacceptable level of toxicity in humans, most likely due to the off-target effects attributable to the inhibition of CA isoforms that are not involved in the target disease or due to general toxic effects of the chemical. Therefore, to target particular therapeutic areas with minimal off-target effects, inhibition of specific enzymes is necessary. Thus, there is a particular need to develop inhibitors that are not only selective against the human CAs but are also safe for clinical use.

Along this line, we have previously developed numerous potent CA inhibitors that are not only effective against specific human α-CAs and mycobacterial β-CAs, but also show minimal toxicity and are suitable for developing further for clinical use as drugs. Recently, we reported sulphonamide and carbamate compounds as hCA IX and mycobacterial β-CA inhibitors. In addition to the synthesis and in vitro and in vivo evaluation of these compounds, we also tested them for safety and toxicity.

Design, synthesis, in vitro inhibition and toxicological evaluation of human carbonic anhydrases I, II and IX inhibitors in 5-nitroimidazole series

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ABSTRACT
With the aim to obtain novel compounds possessing both strong affinity against human carbonic anhydrases and low toxicity, we synthesised novel thiourea and sulphonamide derivatives and studied their inhibitory properties against human CA I, CA II and CA IX. We also evaluated the toxicity of these compounds using zebrafish larvae. Among the three compounds, derivative showed efficient inhibition against hCA II (Ki = 58.6 nM). Compound showed moderate inhibition against hCA II (Ki = 199.2 nM) and hCA IX (Ki = 147.3 nM), whereas it inhibited hCA I less weakly at micromolar concentrations (Ki = 6428.4 nM). All other inhibition constants for these compounds were in the submicromolar range. The toxicity evaluation studies showed no adverse effects on the zebrafish larvae. Our study suggests that these compounds are suitable for further preclinical characterisation as potential inhibitors of hCA I, II and IX.

ARTICLE HISTORY
Received 27 September 2019
Revised 22 October 2019
Accepted 22 October 2019

KEYWORDS
Carbonic anhydrases; carbonic anhydrase inhibitors; synthesis; toxicity evaluation; lethal concentration; zebrafish embryos
using 1–5 d post-fertilisation (dpf) zebrafish larvae. Our studies showed that these inhibitors are safe for further preclinical characterisation using in vivo models. In addition, in this study, we were interested in incorporating a nitro-imidazole moiety as a privileged hypoxia sensitising scaffold in the structure of CA inhibitors. The rationale was to investigate a strategy of dual targeting (hypoxia and hCA IX/hCA XII) in the context of anticancer agents. Previous results have shown the validity of this approach when conjugation of 5-nitroimidazole derivatives to CA inhibitors structures led to new radiosensitiser agents targeting hypoxic tumours. To further investigate the effect and the influence of the 5-nitro imidazole moiety and develop new potent and safer inhibitors, we have been continuing our research on new inhibitors in the nitroimidazole series. In the present study, we report the design, synthesis, in vitro CA inhibition, and evaluation of toxicity of novel thiourea derivatives and a sulphonamide derivative using zebrafish larvae.

**Materials and methods**

**Chemistry**

All reagents and solvents were of commercial quality and used without further purification unless otherwise specified. All reactions were carried out under an inert atmosphere of nitrogen. TLC analyses were performed on silica gel 60 F254 plates (Merck Art. no. 1.05554). Spots were visualised under 254 nm UV illumination or by ninhydrin solution spraying. Melting points were determined using a Büchi Melting Point 510 and are uncorrected. The 1H and 13C NMR spectra were recorded on Bruker DRX-400 spectrometer using DMSO-d6 as solvent and tetramethylsilane as internal standard. NMR (101 MHz, DMSO-d6) δ 8.57 (s, 1H), 7.95 (s, 1H), 7.58 (d, J = 8.5, 2H), 7.20 (d, J = 8.5, 2H), 4.42 (s, 2H), 3.82 (s, 2H), 2.77 (s, 2H), 2.36 (s, 3H). 13C NMR (101 MHz, DMSO-d6) δ 160.49, 133.45, 133.11, 129.40, 118.95, 55.38, 45.93, 43.03, 13.75. MS (ESI+): 349.10 [M + H]+.

**4-(2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethyl) thiourea derivative (3):** The same protocol as for the synthesis of compound 3 starting from 1. Yield: 60%. 1H NMR (400 MHz, DMSO-d6) δ 8.03 (s, 1H), 7.95 (s, 2H), 7.25 (d, J = 8.5, 2H), 7.20 (d, J = 8.5, 2H), 4.42 (s, 2H), 3.82 (s, 2H), 2.77 (s, 2H), 2.36 (s, 3H). 13C NMR (101 MHz, DMSO-d6) δ 151.98, 148.56, 138.56, 137.61, 133.16, 129.77, 121.97, 45.39, 30.63, 13.75. MS (ESI+): 349.10 [M + H]+.

**2-(Methyl-5-nitro-1H-imidazol-1-yl) ethyl methanesulphonate (4):** Methane sulphonylchloride (1.1 equiv.) was added dropwise to a stirred solution of metronidazole, triethylamine (2 equiv.) and DMAP (1.1 equiv.) in anhydrous methylene chloride at 0°C. The reaction mixture was allowed to warm to room temperature and stirred for overnight. The reaction mixture was then diluted with methylene chloride and washed with water and brine. The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated to get crude compound which was used further without purification. Yield: Quantitative. 1H NMR (DMSO-d6, 400 MHz) δ 8.05 (s, 1H), 4.61 (t, J = 7.3, 2H), 3.51 (t, J = 7.3, 2H), 2.50 (s, 3H). 13C NMR (DMSO-d6, 101 MHz) δ 151.08, 138.18, 133.14, 47.09 (s, 1H), 14.09, 2.28. MS (ESI+): m/z 281.97 [M + H]+.

**Potassium thiocacetate (5):** Potassium thiocacetate (2 equiv.) was added to a solution of 1 (1 equiv.) in anhydrous DMF and stirred at room temperature for 16 h. The reaction mixture was then diluted with ethyl acetate and washed with water and brine. The organic layer was dried over anhydrous sodium sulphate, filtered and concentrated under vacuum to give thiocetate. Yield: 83%. 1H NMR (400 MHz, DMSO-d6) δ 8.01 (s, 1H), 4.45 (t, J = 6.6, 2H), 3.27 (t, J = 6.6, 2H), 2.46 (s, 3H), 2.29 (s, 3H). 13C NMR (101 MHz, DMSO-d6) δ 194.59, 151.25, 138.42, 132.92, 44.68, 30.32, 27.87. MS (ESI+): m/z 230.06 [M + H]+.

**2-(Methyl-5-nitro-1H-imidazol-1-yl)ethane-1-sulphonyl chloride (6):** A solution of 7 (1 equiv.) was treated with hydrogen peroxide [35% w/w in water (6 ml)] and acetic acid (7 ml). The reaction mixture was then stirred for 2 h at room temperature. After 1 h an additional 1 ml phosphonic acid solution was added, and the mixture was stirred for 2 h under the same conditions. After precipitation, the reaction mixture was filtered to get pure compound 9 as solid. Yield: 66%. 1H NMR (400 MHz, DMSO-d6) δ 8.80 (s, 1H), 4.63 (t, J = 6.3, 2H), 2.92 (t, J = 6.3, 2H), 2.78 (s, 3H). 13C NMR (101 MHz, DMSO-d6) δ 149.90, 138.05, 123.91, 48.67, 44.17, 12.13. MS (ESI+): m/z 236.00 [M + H]+.

**2-(Methyl-5-nitro-1H-imidazol-1-yl)ethane-1-sulphonamide (10):** To a solution of compound 9 (1 equiv.) in toluene was added at room temperature 0.5 M solution of LiQ ammonia in 1,4-dioxane (2 ml). After 1 h, the reaction mixture was filtered to afford pure

with water. The organic layer was dried over anhydrous magnesium sulphate and concentrated under vacuum. The residue was purified by chromatography on silica gel using methylene chloride-methanol 9:1 as eluent. Yield: 43%. 1H NMR (400 MHz, DMSO-d6) δ 9.35 (s, 1H), 8.03 (s, 1H), 7.51 (s, 2H), 7.11 (d, J = 9.1, 4H), 7.01 (s, 2H), 4.41 (s, 2H), 3.81 (s, 2H), 2.68 (s, 2H), 2.36 (s, 3H). 13C NMR (101 MHz, DMSO-d6) δ 151.98, 139.08, 138.13, 133.70, 133.45–133.11, 129.40, 118.95, 55.38, 45.93, 14.30. MS (ESI+): m/z 428.12 [M + H]+.

4-(2-(2-Methyl-5-nitroimidazol-1-yl)ethyl) thiourea derivative (4):
compound as white solid. Yield: 68%, $^1$H NMR (400 MHz, DMSO-
\text{d}_6) \delta 8.03 (s, 1H), 7.39 (s, 2H), 4.63 (t, J = 7.0, 2H), 3.49 (t, J = 7.0, 2H), 2.49 (s, 3H). $^{13}$C NMR (101 MHz, DMSO-
\text{d}_6) \delta 151.43, 138.36, 133.04, 53.08, 40.85, 13.94. MS (ESI$^+$) m/z 235.05 [M + H]$^+$. 

In vitro inhibition studies
An Applied Photophysics stopped-flow instrument was used for assaying the CA-catalysed CO$_2$ hydration activity$^{21}$. Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer and 20 mM Na$_2$SO$_4$ (for maintaining the ionic strength constant), following the initial rates of the CA-catalysed CO$_2$ hydration reaction for a period of 10–100 s. The CO$_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction were used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water, and dilutions up to 0.01 mM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and represent the average from at least three different determinations. CA isoforms were recombinant ones obtained in house as reported earlier.

Toxicity evaluation

Inhibitors
The three compounds 3, 4 and 10 were designed and synthesised with the purpose of developing them as inhibitors of human CAs. Subsequently, all the three compounds were investigated in vitro as inhibitors of human CA I, CA II and CA IX. These compounds were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to prepare 100 mM stock solutions. Before start of each experiment, the series of dilution were made from the above stock in an embryonic medium [5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$, and 0.1% w/v Methylene Blue (Sigma-Aldrich, Darmstadt, Germany)]. Embryos after 1-day post fertilisation were exposed the each of the diluted inhibitor solution.

Maintenance of zebrafish
AB strains of wild-type adult zebrafish were maintained at 28.5 °C in an incubator$^{19}$. For collecting embryos, 3–5 pairs of male and female fish were set up overnight for breeding$^{22}$. The next morning, from the overnight breeding tanks 1–2 h post fertilisation (hpf) embryos were collected in a sieve and rinsed with embryonic medium [5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, 0.33 mM MgSO$_4$, and 0.1% w/v Methylene Blue (Sigma-Aldrich, Darmstadt, Germany)] and kept the collected embryos in an incubator at 28.5 °C overnight. The toxicity evaluation studies of the inhibitors were performed using the fish that were 24 hpf. All the zebrafish experiments were performed at the zebrafish core facility, Tampere University, Finland and according to the protocol used in our laboratory$^{22}$.

Ethical statement
Tampere University has an established zebrafish core facility authorisation granted by the National Animal Experiment Board (ESAVI/7975/04.10.05/2016). The experiments using developing zebrafish embryos were performed according to the Provincial Government of Eastern Finland Province Social and Health Department Tampere Regional Service Unit protocol # LSLH-2007–7254/Ym-23. Care was taken to ameliorate suffering by euthanizing the 5 dpf larvae by prolonged immersion in a petri dish containing an overdose of Tricaine (Sigma-Aldrich, St. Louis, MO) before fixing in buffered formaldehyde for histochemical analysis.

Determination of LC$_{50}$
The LC$_{50}$ values of the compounds 3, 4 and 10 were determined using 1-dpf embryos exposed to 10–12 different inhibitor concentrations. For each concentration of the inhibitor, we used 30 1dpf larvae$^{15,22}$. In each group, the larvae were exposed to different concentrations of the inhibitors that ranged from 12.5 μM to 3 mM. The dose response curve (DRC) was calculated using DRM of the DRC R package$^{22}$. The control group was constituted of an equal number of larvae not treated with any chemical and the larvae that were treated with 1% of DMSO. Toxicological evaluation studies were performed in 24-well plates (Corning V R Co-star V R cell culture plates). In each well, we placed 1–2 1-dpf embryos in 1 ml of embryonic medium containing a diluted inhibitor. For control groups, 1% DMSO diluted in either embryonic medium or embryonic medium with no chemical compound. A minimum of three sets of experiments were carried out for each inhibitor. The mortality of the larvae was checked every 24 h until 5 d after exposure to the inhibitors.

Phenotypic analysis of control and inhibitor treated larvae
We assessed the effect of inhibitors on the zebrafish larvae after 5d of exposure to the drugs; we analysed eight observable phenotypic parameters: (1) mortality, (2) hatching, (3) oedema, (4) movement pattern, (5) yolk sac utilisation, (6) heartbeat, (7) body shape and (8) swim bladder development, using a stereo microscope and recorded the observations for each inhibitor treated and control groups. The images of the developing larvae were taken using a Lumar V12 fluorescence microscope attached to a camera with a 1.5 lens (Carl Zeiss Microlmaging GmbH, Göttingen, Germany). The images were analysed with AxioVision software versions 4.7 and 4.8 as described in our standard protocol for assessment of toxicity and safety of the chemical compounds$^{22}$.

Swim pattern analysis
The movement of the zebrafish larvae was tracked from day 4 of exposure to these inhibitors. Similarly, detailed analysis of the swim pattern of the larvae was performed at the end of day 5 after exposure to the inhibitors. For analyses of movement pattern, about 10–15 the zebrafish larvae were placed in a 35 mm × 15 mm petri dish containing embryonic medium and the larvae were allowed to settle in the petri dish for 1 min. The movement of the zebrafish larvae was observed under the microscope for 1 min. A short video of about 1 min was taken for each group of larvae that were treated with 125 μM concentration. The swim patterns were compared with the control group zebrafish larvae that were not treated with any inhibitor.
Histological studies

The histological analyses were done to check the effect of inhibitors on the internal tissues of the larvae that were treated with different concentrations of the inhibitors used in the study and the control group larvae not treated with any inhibitor and the larvae treated with 1% DMSO. After 5 d of exposure to different concentration of the inhibitors, the larvae were washed with phosphate buffered saline (PBS) immersed in excess amounts of Tricaine to anaesthetize the larvae. The larvae thus prepared were transferred to a 1.5 ml microcentrifuge tube and fixed in buffered formaldehyde (formaldehyde solution 4%, pH 6.9) in PBS for 3 h at room temperature or overnight at 4°C. After the fixation, the larvae were transferred to 70% ethanol and stored at 4°C before being embedded in paraffin. The paraffin-embedded samples were sectioned into 5 μm thin slices for the histochemical staining. The fixed sections containing samples were deparaffinised in xylene, rehydrated in an alcohol series, and stained with Mayer’s Haematoxylin and Eosin Y (both from Sigma-Aldrich, Darmstadt, Germany). After dehydration, the slides were mounted with Entellan® Neu (Merck; Darmstadt, Germany). The slides containing the tissues were examined for morphological changes in the tissues of the larvae exposed to the drugs and photographed using a Nikon Microphot microscope (Nikon Microphot FXA, Tokyo, Japan). All the procedures were carried out at room temperature unless stated otherwise.

Statistical analysis

The GraphPad Prism software (5.02) (GraphPad Software, La Jolla, CA) was used to perform some of the statistical analyses. For statistical analysis of the toxicity parameters, a two-tailed Fisher’s test was used and p values below 0.05 were considered significant.

Results and discussion

Chemistry

Thiourea derivatives 3 and 4 were prepared starting from the amino analogue of metronidazole 1 which was converted to isothiocyanate 2 using thiophosgene under basic conditions. Reaction either with 4-(2-aminoethyl) aniline or 4-(2-aminoethyl) phenol in acetonitrile led to the thiourea-derivatives which were then sulphamoylated with sulphamoyl chloride to give, respectively, sulphamide 3 and sulphamate 4 (Scheme 1).

- Compound 10 was prepared in a five-step synthesis starting from metronidazole 5. Mesylation of 5 using methane sulphonyl chloride under basic conditions led to compound 6. Reactions with potassium thioacetate followed by oxidation and treatment with phosgene allowed the formation of sulphonyl chloride 9.
- Finally, compound 10 was obtained by treatment with a solution of ammonia in 1,4-dioxane at room temperature (Scheme 2).

CA inhibition assay

All compounds reported here were assayed as inhibitors of three physiologically relevant CA isoforms, the cytosolic hCA I and II and the transmembrane, tumour-associated hCA IX using the CO₂ hydrase assay (Table 1). The clinically employed sulphonamide, acetazolamide (AAZ, 5-acetamido-1,3,4-thiadiazole-2-sulphonamide), has been used as a standard in these measurements.

- Compound 10 showed very weak inhibition potency against the abundant cytosolic hCA I with a Kᵢ value of 6428 nM. The thiourea derivatives 3 and 4 demonstrated sub-micromolar inhibition potency against all CA isoforms. Compound 4 showed the most efficient binding to hCA II (Kᵢ = 58.6 nM) with a selectivity ratio of 0.09 for inhibiting hCA II over hCA IX, explained by the influence of the substitution on the benzene ring. Compound 10, the sulphonamide derivative of the sulphamide and sulphamate analogues previously reported and biologically evaluated by our group, showed moderate binding efficacy towards hCA II (Kᵢ = 199.2 nM) and hCA IX (Kᵢ = 147.3 nM). The better binding efficacy of sulphonamide 10 compared to the sulphamide and sulphamate compounds might be explained by higher stability under acidic conditions, i.e. sulphonamide > sulphamide > sulphamate.

Evaluation of safety and toxicity of compounds 3, 4 and 10

Determination of inhibitor half maximal lethal concentration (LC₅₀)

The lethal effects of all three inhibitor compounds were concentration dependent on the developing zebrafish embryos that were exposed to the inhibitor for 5 d (Figure 1). None of the three inhibitors exhibited any significant mortality in up to 1.5 mM.
at submillimolar range. Previous toxicity studies on hCA IX and inhibitors on different parameters in treated larvae. The results compounds in zebrafish larvae across the concentration range m

were 3 mM for concentrations and the embryos showed normal phenotypes observable changes in the developing zebrafish embryos at these larvae. The microscopic examination did not show any of the

ranging from 75

sentative larvae exposed to the compounds with concentrations or treated with 1% DMSO. The images in Figure 2 show the repre-

sentative video of swim pattern treated with com-

pound

3

10

: Supplementary Appendix A). The results of the current study reveal that the larvae treated with 3, 4 and 10 become slower while swimming as compared to the swim pattern of the control group larvae. However, swim pattern of larvae treated with 3, 4 and 10 was unlike the larvae treated with nitroimidazole-based inhibitors DTP338 and DTP348, which showed ataxic movement pattern due to neurotoxicity at a concentration as low as 100 μM. Therefore, these novel thiourea and sulphonamide derivatives can be considered safer for further preclinical charac-

terisation and development as potential drugs.

### Histochemical analysis

The effects of different concentrations of CA inhibitors on histology were studied by analysing the sections of 5 dpf zebrafish larvae stained with haematoxylin and eosin (H & E). The stained sagittal sections of both the inhibitor treated and control larvae were compared by light microscopy. Representative images of zebrafish larval sections are shown in Figure 4. The histological examination revealed no damage to the tissues of the developing zebrafish larvae. Even high concentrations (up to 2 mM) showed no apparent damage to the internal tissues, suggesting that these compounds are safe and can be further developed as CA-specific inhibitors for clinical use.

### Analysis of swim pattern

During development, zebrafish embryos are easily affected by chemical compounds compared to adult zebrafish or other animal or cell models, and therefore are suitable for assessing the subtle toxic effects of the chemicals. To assess the subtle toxic effects of the inhibitors, the swim pattern of the larvae was examined under the microscope during treatment with the CA inhibitors. The swim pattern analysis showed no abnormal or ataxic movement pattern in the larvae exposed to the inhibitors, up to 2 mM concentration. However, a detailed analysis of the videos at the end of 5 d of inhibitor exposure showed that the larvae of the inhibitor treated groups (125

m

and

10

were as active and tended to remain at the sides of petri dish compared to the control group larva. We further assessed the toxicity of compounds 3, 4 and 10 compounds in zebrafish larvae across the concentration range 12 μM–3 mM. Figure 3 presents plots of dose-dependent effects of inhibitors on different parameters in treated larvae. The results indicated that none of the inhibitors show significant toxic effects at submillimolar range. Previous toxicity studies on hCA IX and

mycobacterial β-CA inhibitors showed significant effects on larval mortality and observable phenotypic parameters in the concentration range of 50–300 μM. Therefore, our present results show that 3, 4 and 10 are less toxic in the zebrafish larval model.

### Phenotypic analyses of zebrafish larvae treated with inhibitor compounds

To assess toxic effects of the inhibitors on the developing zebrafish larvae we analysed eight observable phenotypic parameters namely: hatching, heartbeat, yolk sack utilisation, movement pattern, body shape, swim bladder development, mortality and oedema. The parameters were monitored using a stereo microscope and observations were recorded for each group in comparison with control embryos that were not treated with any of the compounds or treated with 1% DMSO. The images in Figure 2 show the representative larvae exposed to the compounds with concentrations ranging from 75 μM to 500 μM as compared to the control group larvae. The microscopic examination did not show any of the observable changes in the developing zebrafish embryos at these concentrations and the embryos showed normal phenotypes throughout the period of embryonic development from 2 to 5 dpf.

The effects of different concentrations of hCA I, hCA II and hCA IX using a stopped flow CO2 hydrase assay.

| Compound | hCA I (nM) | hCA II (nM) | hCA IX (nM) | Selectivity ratios |
|----------|------------|-------------|-------------|-------------------|
| 3        | 152.2      | 612.8       | 290.6       | 2.10              |
| 4        | 127.8      | 58.6        | 642.6       | 0.09              |
| 10       | 6428.4     | 199.2       | 147.3       | 1.35              |
| AAZ      | 250        | 12.0        | 25          | 0.48              |

*Average from three different assays, by a stopped flow technique (errors were in the range of ± 5–10% of the reported values).

**Table 1.** Inhibition data of compounds 3, 4 and 10 against hCA I, hCA II and hCA IX using a stopped flow CO2 hydrase assay.

**Scheme 2.** Synthesis of sulphonamide 10. Reagents and conditions: (i) CH3SO2Cl, NEt3, DMAP, DCM, 0°C – rt; (ii) K2S2O4, DMF, rt; (iii) 35% H2O2–CH2COOH, rt; (iv) phos-

gene, cat. dry DMF, dry DCM, rt. (v) NH3 in 1,4 dioxane, benzene, rt.

Concentration of the inhibitors were higher compared to any tox-

icity and safety assessment studies that we have performed ear-

lier, suggesting that these compounds can be further developed as inhibitors of human CA I, CA II and CA IX.

Concentration at the end of 5 d of exposure to the inhibitors. Significant mortality of zebrafish larvae emerged at higher concentrations (>2 mM), and the LC50 values at the end of five days were 3 mM for 3 and 2.6 mM for 4 and 10. The half maximal lethal concentrations of the inhibitors were higher compared to any toxicity and safety assessment studies that we have performed earlier, suggesting that these compounds can be further developed as inhibitors of human CA I, CA II and CA IX.
Figure 1. The LC50 values of the tested CA inhibitor compounds. The LC50 doses for the inhibitors were determined based on the 50% mortality of the larvae at the end of 5 d after the exposure of embryos to different concentrations of inhibitors: compound 3 (A), 4 (B) and 10 (C). The LC50 doses were determined after three independent experiments with similar experimental conditions (for each compound N = 90).

Figure 2. The images of zebrafish larvae in control and inhibitor treated groups. Representative images of 2–5 dpf zebrafish larvae exposed to different concentrations (75, 250 and 500 µM) of compound 3, 4 and 10. The images of control group (not treated with inhibitors) and 1% DMSO treated zebrafish larvae show normal development (n = 60).
Figure 3. The effect of compounds 3, 4 and 10 CA inhibitors on the phenotypic parameters of the 5 dpf zebrafish larvae. The plot graphs of different phenotypic abnormalities in (A) hatching, (B) oedema, (C) heartbeat (D) body shape and (E) yolk sac utilisation. For each concentration, \( n = 90 \). *\( p < 0.05 \) by two-tailed Fisher’s test.

Figure 4. Histochemical analyses of zebrafish larvae. Representative sagittal sections of an untreated zebrafish larva (A) or larva treated with 500 \( \mu \text{M} \) of compound 3 (B), 4 (C) or 10 (D). The images presented here are selected from three independent groups of experiments.
Conclusions
In conclusion, we have designed and synthesised thiourea and sulphonamide derivative compounds with the potential to inhibit human CA I, CA II and CA IX. These proteins which play important pathophysiological roles in human diseases, such as well-known role of CA IX in the progression of solid tumours, and those of CA I and CA II in retinal and cerebral oedema, glaucoma, epilepsy and altitude sickness. The inhibition kinetics of these inhibitors were studied against recombinant human hCA I, hCA II and hCA IX in vitro. Among these compounds, 4 was the most efficient against hCA II (K1 58.6 nM). 10 inhibited human CA IX with K1 147.3 nM. The inhibition properties of these inhibitors against the three hCA isoforms prompted us to test these drugs further for their safety in a zebrafish larval model.

The toxicological evaluation indicated that these inhibitors showed no adverse morphological changes nor did they cause abnormal swim pattern at any sub-millimolar concentration. Taken together, the results from both in vitro inhibition studies and toxicological evaluation, we suggest that these inhibitors could be developed into potential drugs targeting hCA I, hCA II and hCA IX with no, or minimal, off-target effects.

Acknowledgements
The authors thank Aulikki Lehmus and Marianne Kuuslahti for the histochemical analyses of the zebrafish samples. We also thank Leena Mäkinen and Hannaleena Piippo for the assistance with zebrafish embryos for the experiments.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
The work was supported by grants from Sigrid Jusélius Foundation (SP, MP), Finnish Cultural Foundation (HB, AA), Academy of Finland (SP), and Jane & Aatos Erkko Foundation (SP). Authors acknowledge financial support from ERC advanced grant (ERC-ADG-2015, no. 694812 – Hypoximmuno).

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