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Study of Chalcogen Aspirin Derivatives with Carbonic Anhydrase Inhibitory Properties for Treating Inflammatory Pain

Elena Lucarini, Vanessa D'Antogiovanni, Luca Antonioli, Carla Ghelardini, Lorenzo Di Cesare Mannelli, Marta Ferraroni, Maria Locuoco, Antonella Capperucci, Damiano Tanini,* Andrea Angeli,* and Claudiu T. Supuran

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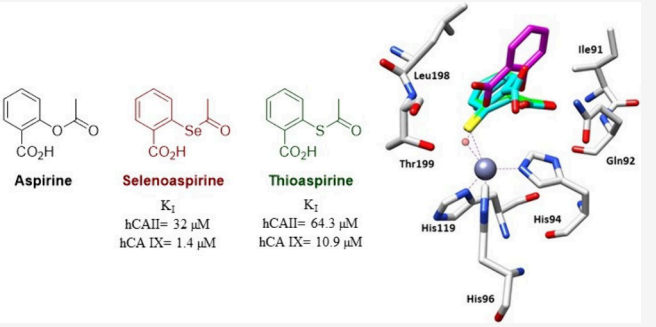
ABSTRACT: Carbonic anhydrase (CA) inhibitors represent intriguing tools for treating pain. This study aims at studying the pharmacological profile of chalcogen bioisosteres of aspirin, as inhibitors of CA isoforms (hCA I, II, IV, VII, IX, and XII). Our results show that selenoaspirin (**5**) displayed markedly superior inhibitory potency across all tested isoforms compared to thioaspirin (**7**) and aspirin, with a strong selectivity against the isoform CA IX. X-ray crystallography confirmed that both compounds bind effectively within the active site of hCA II, revealing unique structural characteristics compared to those of aspirin. In a preclinical model of inflammatory pain, compound **7** exhibited a longer lasting antihyperalgesic effect than aspirin, though with a lower potency. Conversely, compound **5** exhibited both lower potency and efficacy than aspirin in reducing pain, which entailed both adverse effects. Nevertheless, the therapeutic potential of chalcogen-based aspirin derivatives as novel CA inhibitors deserves to be further explored for clinical applications.



KEYWORDS: *Carbonic anhydrase, metalloenzyme, aspirin, selenium, inflammatory pain*

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical settings, with aspirin being the most popular for its anti-inflammatory and analgesic properties.[1] Although NSAIDs vary in chemical structure, their therapeutic effects stem from inhibiting cyclooxygenase (COX) isoforms, which block prostaglandin biosynthesis.[2] Over recent decades, several modifications have been made to NSAID scaffolds to retain their anti-inflammatory efficacy while enhancing gastrointestinal tolerance or incorporating immune-modulating and antioxidant activities.[3−5] Particularly, chalcogen isosteric replacements have garnered significant interest as they offer safer, more effective drug candidates with improved biopharmacological profiles compared to the original compounds.[6−9] Despite the similar physicochemical properties of chalcogens, they exhibit varying chemical reactivity due to differences in acidity, redox potential, electrophilicity, and nucleophilicity. These differences primarily arise from the larger size and greater polarizability of the selenium atom compared to sulfur.[10] Adding chalcogen groups to NSAIDs has resulted in many analogs with notable biological activities. Therefore, combining NSAIDs with chalcogen moieties is a promising approach to developing new clinical treatments for various diseases,[11] balancing reactive oxygen species (ROS) levels, and enhancing antioxidant effects.

The high concentration of hydrogen ions in various inflammatory diseases has been identified as a crucial link between inflammation and pain.[12] One significant biochemical process contributing to proton generation in resting tissues is the reversible hydration of carbon dioxide, mediated by carbonic anhydrase (CA) enzymes.[13−15] Different CA isoforms have been implicated in various inflammatory disorders.[16−18] Notably, CA IX and XII are frequently found in hypoxic tumors and are overexpressed in the inflamed synovium of patients with juvenile idiopathic arthritis.[19] Additionally, CA IV, which is abundantly present in the intestine, plays a significant role in healing intestinal damage and developing persistent visceral pain.[20] Recent studies have shown that aspirin binds to human CA II, and this isoform is overexpressed in patients with aspirin resistance, possibly making it an unidentified carboxylesterase.[21] Given this information, developing chalcogen-based aspirin bioisosteres as new CA-

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NSAID inhibitors appears to be a promising strategy. In this study, we present the design and synthesis of selenium and thioaspirin analogs, their CA inhibition profiles, and binding modes against hCA II through X-ray crystallography, along with a preliminary assessment of their antihyperalgesic effects.

Having in mind the structure of selenium- and sulfur-containing isosteres of aspirine (Figure 1), their synthesis was

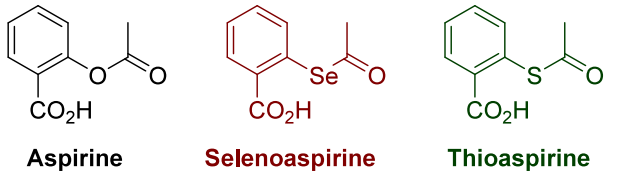
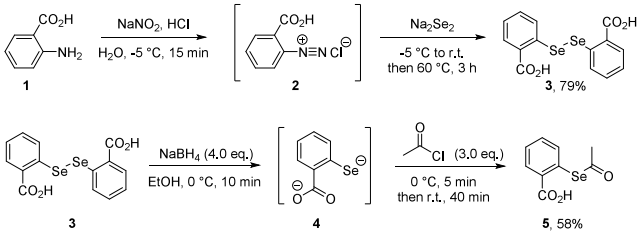


Figure 1. Molecular structure of aspirine and its sulfur- and selenium-containing analogues.

then pursued. On the basis of our previously reported studies on selenolesters[22,23] and considering the reactivity of diselenides,[24,25] we envisaged the diselenide **3** as a suitable precursor or the preparation of selenoaspirine.

The diselenide **3** (diseleno salicylic acid), which is the intermediate most commonly employed for the synthesis of ebselen, was prepared via diazonium salt according to the literature.[26] Upon treatment with $NaNO_2$/HCl, anthranilic acid **1** readily underwent diazotisation to provide the corresponding diazonium salt **2**, which was reacted *in situ* with a freshly prepared solution of sodium diselenide affording the diselenide **3** in good yield (Scheme 1). Reductive cleavage

Scheme 1. Synthetic Route for the Synthesis of Selenoaspirine 5 from Anthranilic Acid 1

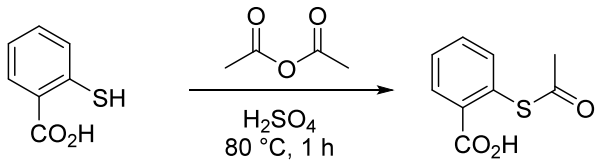


of **3** represents a direct route toward the selenolate **4**, which, in principle, can be either protonated to provide the corresponding selenol or functionalized *in situ* with suitable electrophiles. Notably, attempts to isolate the selenol form of **4** (selenosalicylic acid) using our previously reported procedure[27,28] were unsuccessful and gave only low yield of the desired selenol, in mixture with the corresponding diselenide **3**. On the other hand, the selenolate **4**, easily achieved upon reduction of **3** with $NaBH_4$, was selectively Se-acetylated *in situ* upon treatment with acetyl chloride to provide the desired selenoaspirine **5**.

The synthesis of thioaspirine **7** was easier, as it could be straightforwardly obtained in good yield from commercially available thiosalicilic acid **6**, via S-acylation with acetic anhydride in the presence of sulfuric acid in catalytic amount (Scheme 2).[29,30]

Compounds **5**, **7**, and **aspirin** were profiled *in vitro* against the physiological relevant human (h) isoforms hCA I, II, IV, VII, IX, and XII by applying the stopped-flow technique and were compared with the standard sulfonamide inhibitor acetazolamide (**AAZ**) (Table 1).

Scheme 2. Synthesis of Thioaspirine 7 from Thiosalicylic Acid 6



6 **7**, 72%

Based on the data presented in Table 1, the structure–activity relationship (SAR) of the tested compounds can be summarized as follows.

Aspirin, in general, did not exhibit any significant activity against the tested isoforms. This aligns with previous studies, which reported weak inhibitory potency with an IC_{50} of 5.5 mM.[32] However, replacing the oxygen with chalcogen atoms such as selenium or sulfur significantly enhanced the potency against CAs here studied, likely due to the easier hydrolysis of chalcogen esters compared to aspirin. This pattern is also evident between thioaspirin (**7**) and selenoaspirin (**5**), where the latter proved to be a more effective inhibitor across all isoforms, probably for the same reasons mentioned for aspirin. For the most abundant isoforms, hCA I and II, compound **5** demonstrated approximately 14-fold greater activity than compound **7** against hCA I (K_I 5.2 and 72.5 μM, respectively) and 2-fold greater activity against hCA II (K_I 32.0 and 64.3 μM, respectively). In the case of the cytosolic isoform CA VII, potency was observed to increase 3-fold when moving from thioaspirin (**7**) to selenoaspirin (**5**). Regarding membrane-bound isoforms, CA IV exhibited the least difference in potency between chalcogen aspirins (K_I = 48.0 μM for **5** and 77.3 μM for **7**). However, derivative **5** showed strong selectivity against the tumor-associated isoform CA IX, with nearly a 10-fold increase in potency (K_I 1.4 μM and 10.9 μM, respectively).

Previous studies involving chalcogen inhibitors, combined with the knowledge that CAs possesses both thio- and seleno-esterase activities,[22,32,33] prompted us to explore how new aspirin analogs **5** and **7** bind within the CAII active site using of X-ray crystallography. Analysis of the data revealed the hydrolysis of the chalcogen esters, with the resulting selenol and thiol bound to the CAII active site instead of compounds **5** and **7** (Figures 2 and S1). The hydrolyzed form of selenoaspirin (**5**) was found to bind directly to the zinc ion, displacing the bound solvent molecule via the same mechanism previously reported for this class of CA inhibitors (Figure 2A).[27,34]

This compound is stabilized within the active site through interactions with residues on both the hydrophobic and hydrophilic faces. On the hydrophilic pocket, residue Thr200 forms a hydrogen bond with the carboxylic acid group of the hydrolyzed form of compound **5**. Additionally, the residues Gln92, Asn67, and Asn62 create a network of water bridges with the carboxylic moiety of the derivative stabilizing the complex. On the hydrophobic pocket, residues Val121, Leu141, and Leu198 engage multiple van der Waals interactions with the aromatic ring of the hydrolyzed form of compound **5** (Figure 2A). The second inhibitor analyzed, the thioaspirin **7**, displayed a binding mode similar to that of thiophenol[35] and was observed in a dual conformation within the hCA II active site. Notably, in one of the two

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Table 1. Inhibition Data[a] of Catalytically Active hCA Isoforms I, II, IV, VII, IX, and XII with Compounds 5, 7, Aspirin, and AAZ by a Stopped-Flow CO_2 Hydrase Assay[31]

	KI (μM)					
Cmp	hCA I	hCA II	hCA IV	hCA VII	hCA IX	hCA XII
Aspirin	>100	>100	>100	>100	>100	>100
5	5.2	32.0	48.0	5.2	1.4	8.2
7	72.5	64.3	77.3	18.5	10.9	14.0
AAZ	0.25	0.012	0.074	0.003	0.026	0.006

[a]Mean from 3 different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values).

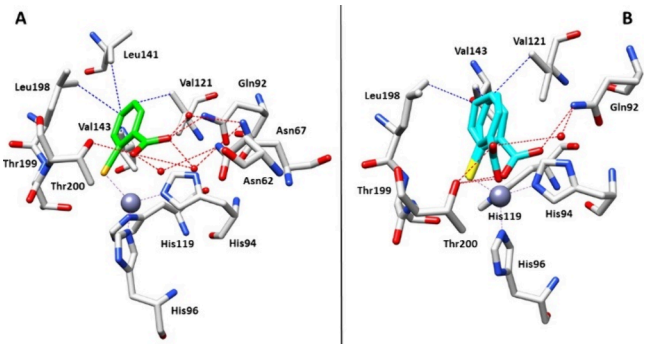

Figure 2. A) Active site region of hCA II/hydrolyzed form of **5** adduct (PDB: 9FAI). Inhibitor is labeled in green, van der Waals interactions in blue, and water bridges as well as hydrogen bonds in red. B) Active site region of hCA II/hydrolyzed form of **7** adduct (PDB: 9FAO). Inhibitor is labeled in cyan, van der Waals interactions in blue, and water bridges as well as hydrogen bonds in red.

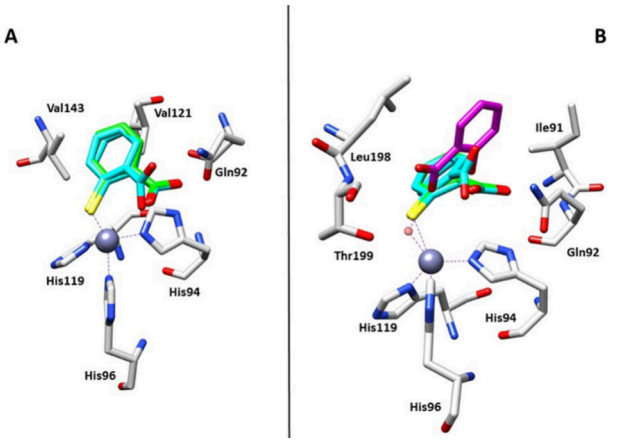

Figure 3. A) Overlay of the hydrolyzed form of selenoaspirin **5** (green) and thioaspirin **7** (cyan) with hCA II. B) Overlay of the hydrolyzed form of aspirin (magenta, PDB: 6UX1), selenoaspirin **5** (green), and thioaspirin **7** (cyan) with hCA II. Specific residues are labeled.

conformations, the carboxylic moiety of the hydrolyzed form of compound **7** exhibited a 70° rotation, likely to facilitate multiple hydrogen bonds with Thr200 (Figure 2B). Furthermore, a water bridge formed between the carboxylic moiety of the hydrolyzed form of compound **7** and Gln92 stabilized the inhibitor within the active site. The hydrophobic pocket of hCA II also engages in van der Waals interactions with the aromatic ring of the inhibitor through residues Val121 and Leu198 (Figure 2B). The overlap of the hydrolyzed form of seleno- and thio-analogs of aspirin reveals a similar binding mode, with distances from the zinc ion of 2.24 Å for compound **7** and 2.42 Å for derivative **5** due to the greater dimension of Se compared to S (Figure 3A). When these two compounds are superimposed with aspirin,[32] their distinct binding modes and locations within the active site of hCA II become evident. These differences can be attributed to the significantly higher nucleophilicity of thio- and selenolate ions compared to phenols, owing to the greater polarizability of chalcogen atoms. Notably, unlike the hydrolyzed form of compounds **5** and **7**, the carboxylic acid moiety in aspirin is anchored to the bound solvent molecule and does not participate in the network of hydrogen bonds within the hydrophilic pocket of the active site (Figure 3B).

As promising candidates for the treatment of inflammatory pain, compounds **5** and **7** were evaluated for their analgesic properties in a preclinical model of disease induced by the intraplantar injection of CFA in a mouse hindlimb paw. The pain relieving efficacy of these compounds was investigated in comparison to that of aspirin by Hot Plate test, Paw Pressure test, and Von Frey test, the results of which were summarized in Figure 4−6.

Twenty-four hours after the injection of CFA, mice developed thermal hyperalgesia (Hot plate test; Figure 4), as well as mechanical hyperalgesia (Paw Pressure test; Figure 5) and allodynia (Von Frey test; Figure 6). In all tests, aspirin showed a dose-dependent antihyperalgesic effect starting from the dose 50 mg kg^{-1} and reaching the highest efficacy at dose of 200 mg kg^{-1}. The effect peaked 40 min after the oral administration of aspirin, then progressively decreased end expired at 80 min (Figures 4A, 5A, and 6A).

Compound **7** showed the same antihyperlagesic efficacy of aspirin but with a lower potency; in fact, the compound was partially effective at the dose of 220 mg kg^{-1} (equimolar to aspirin 200 mg kg^{-1}) but reverted thermal hypersensitivity in CFA animals at the dose of 330 mg kg^{-1}. Interestingly, the effect of the highest dose of compound **7** displayed a longer duration of action compared to the reference drug (Figures 4B, 5B, and 6B). Indeed, compound **7** administrations significantly increased animal pain threshold with respect to pretest starting from 20 min up to 80 or 100 min (according to the test), making it an interesting starting point for developing new derivatives with long-lasting effects.

On the other hand, compound **5** showed both a lower efficacy and a lower potency than the reference compound aspirin on pain evoked by mechanical stimuli in CFA-treated mice (Figures 5C and 6C; Paw Pressure test and Incapacitance test, respectively). Indeed, compound **5**, administered at a dose of 270 mg kg^{-1} (equimolar to aspirin 200 mg kg^{-1}), significantly increased mice pain thresholds only after 40 min with a limited efficacy (about 50%). The same dose of compound **5** was also partially effective on thermal hyperalgesia associated with CFA-related inflammatory pain (Figure 4C). Augmenting the dose (405 mg kg^{-1}) did not result in a

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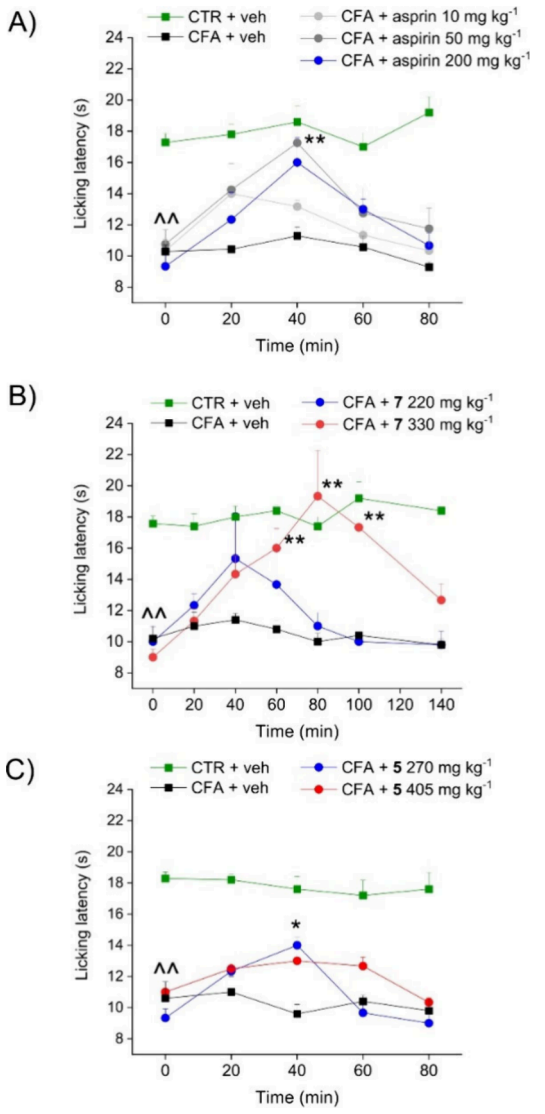

Figure 4. Effect of acute administration of aspirin and compounds **5** and 7 on thermal hyperalgesia associated with inflammatory pain in CFA-injected mice. Inflammatory pain was induced by intraplantar injection of CFA. Control animals were treated with vehicles. Thermal hyperalgesia was evaluated by Hot Plate test, pain-related behavior (i.e., licking of the hind paw) was observed, and the time (seconds) of the first sign was recorded. Twenty-four hours after CFA injection, **aspirin** (10−200 mg kg^{-1}; MW 180.16; A), 7 (220−330 mg kg^{-1}; MW 196.22; B), and **5** (270−405 mg kg^{-1}; MW 243.13; C) were orally administered and measurements assessed before and every 20 min after injection. ^^$p < 0.01$ vs CTR + vehicle treated animals. *$p < 0.05$ and **$p < 0.01$ vs pretest (0 min). Each value represents the mean of 6 mice performed in 2 different experimental sets.

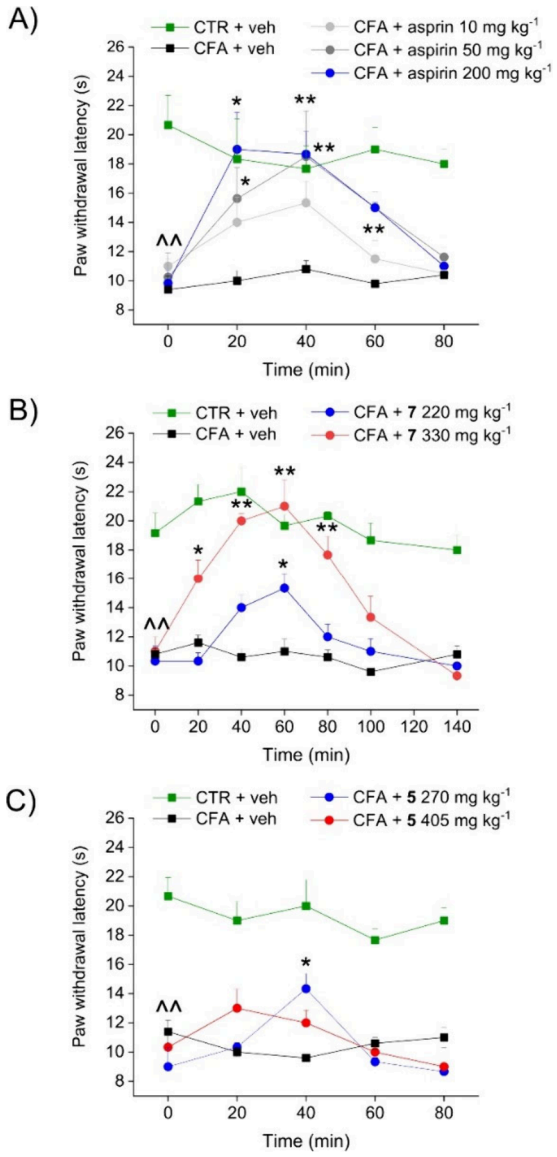

Figure 5. Effect of acute administration of aspirin and compounds **5** and 7 on mechanical hyperalgesia associated with inflammatory pain in CFA-injected mice. Inflammatory pain was induced by the intraplantar injection of CFA. Control animals were treated with vehicles. Mechanical hyperalgesia was evaluated by the Paw Pressure test, and data are expressed as the latency of animal paw withdrawal (s). Twenty-four hours after CFA injection, **aspirin** (10−200 mg kg^{-1}; MW 180.16; A), 7 (220−330 mg kg^{-1}; MW 196.22; B), and **5** (270−405 mg kg^{-1}; MW 243.13; C) were orally administered and measurements assessed before and every 20 min after injection. ^^$p < 0.01$ vs CTR + vehicle treated animals. *$p < 0.05$ and **$p < 0.01$ vs pretest (0 min). Each value represents the mean of 6 mice performed in 2 different experimental sets.

gain in pharmacological efficacy, but rather in the onset of side effects, which impeded to properly carry out Von Frey test (Figure 6C).

Long-term treatment with nonsteroidal anti-inflammatory drugs is associated with gastrointestinal side effects.[36] Aspirin has been shown to inhibit cell proliferation in human intestinal epithelial cell lines[37,38] as well as to induce gastric epithelial barrier dysfunction.[39] The effect of compound 7 on intestinal epithelial barrier integrity and permeability was investigated by measuring the paracellular flux by FITC-dextran fluorescence across Caco-2 cell monolayers (Figure 7). Our results showed a significant increase in FITC-dextran fluorescence in the lower compartment of the trans-well insert in Caco-2 cells treated with both aspirin and compound 7, thus suggesting that both compounds induced an impairment in Caco-2 monolayer integrity.

In summary, this study explored the analgesic properties and CA inhibitory effects of novel chalcogen analogues of **aspirin**, seleno- (**5**), and thioaspirin (7) to better understand their potential for treating inflammatory pain. The results demonstrate that both compounds exhibited promising

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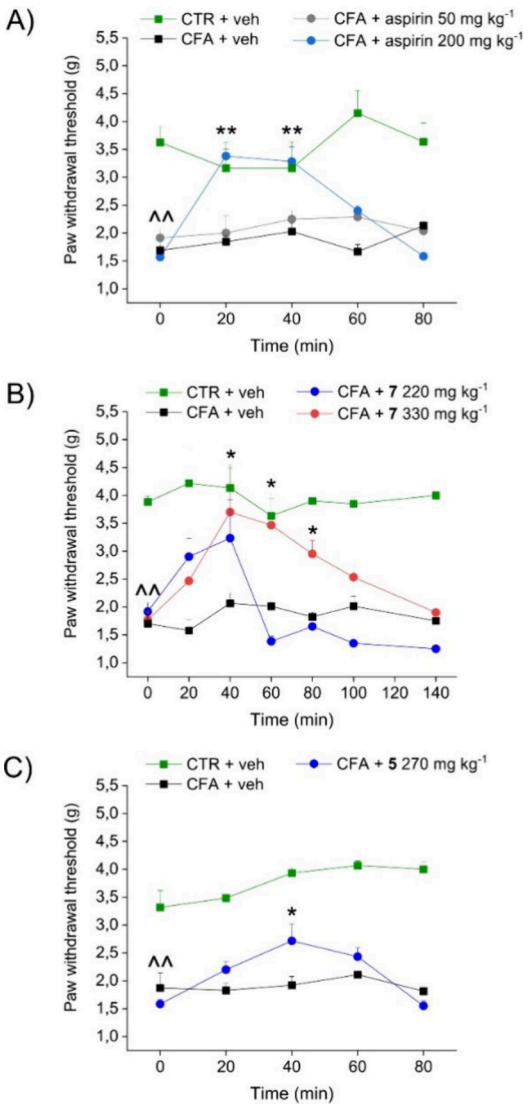

Figure 6. Effect of acute administration of **aspirin**, **5**, and **7** on mechanical allodynia associated with inflammatory pain in CFA-injected mice. Inflammatory pain was induced by the intraplantar injection of CFA. Control animals were treated with vehicles. Mechanical allodynia was evaluated by the Von Frey test, and data are expressed as the weight (g) that the animal tolerated on the paw. Twenty-four hours after CFA injection, **aspirin** (50−200 mg kg^{-1}; MW 180.16; A), **7** (220−330 mg kg^{-1}; MW 196.22; B), and **5** (270 mg kg^{-1}; MW 243.13; C) were orally administered and measurements assessed before and every 20 min after injection. $^{\wedge\wedge}p < 0.01$ vs CTR + vehicle treated animals. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs pretest (0 min). Each value represents the mean of 6 mice performed in 2 different experimental sets.

inhibitory activity against several CA isoforms, though with notable differences in potency and selectivity, and X-ray crystallography showed that both compounds bind effectively within the active site of hCA II. Notably, compound **5** proved to be particularly potent, showing significant inhibition of the tumor-associated CA IX isoform with nearly a 10-fold increase in potency compared to thioaspirin **7**. It also displayed strong activity against the most abundant isoforms, hCA I and II, making it a promising candidate for targeted inhibition. However, derivative **5** efficacy and potency were less notable when its efficacy against mechanical and thermal hyperalgesia was evaluated in a model of inflammatory pain. Conversely,

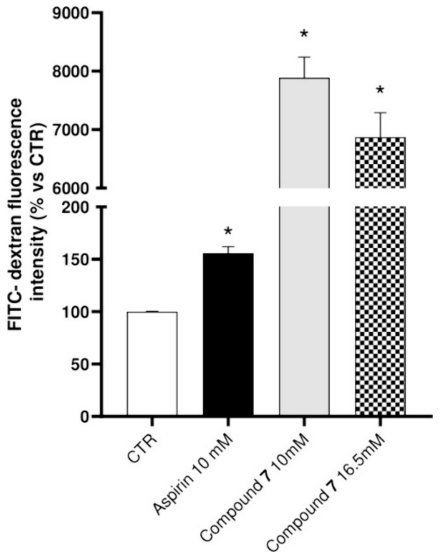

Figure 7. Effect of aspirin and compound **7** on trans-epithelial permeability. FITC-dextran fluorescence intensity measured in Caco-2 cells treated with aspirin (10 mM) or compound **7** (10 mM and 16.5 mM) for 72 h. Each column represents the mean ± SEM ($n = 5$ independent experiments). $^{*}P < 0.05$ significant difference versus control cells (CTR).

derivative **7** displayed less selectivity for specific CA isoforms compared to selenoaspirin **5** but still demonstrated comparable inhibitory effects across a broader range of isoforms. Despite its lower potency overall, thioaspirin **7** effectively relieved the inflammatory pain in CFA-treated mice. Noteworthy, this compound showed a longer duration of action compared to aspirin and selenoaspirin **5**. Further research should focus on refining their structures and assessing their clinical efficacy in other preclinical models.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.4c00284.

Experimental sections, NMR spectra, and X-ray data collection (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Damiano Tanini − *Department of Chemistry "Ugo Schiff" (DICUS), University of Florence, I-50019 Florence, Italy;* orcid.org/0000-0001-8930-3566; Email: damiano.tanini@unifi.it

Andrea Angeli − *NEUROFARBA Department, Sezione di Scienze Farmaceutiche, University of Florence, 50019 Florence, Italy;* orcid.org/0000-0002-1470-7192; Email: andrea.angeli@unifi.it

Authors

Elena Lucarini − *Department of Neuroscience, Psychology, Drug Research and Child Health, Neurofarba, Pharmacology and Toxicology Section, University of Florence, 6-50139 Florence, Italy*

Vanessa D'Antogiovanni − *Department of Clinical and Experimental Medicine, University of Pisa, 56126 Pisa, Italy*

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Luca Antonioli − *Department of Clinical and Experimental Medicine, University of Pisa, 56126 Pisa, Italy*
Carla Ghelardini − *Department of Neuroscience, Psychology, Drug Research and Child Health, Neurofarba, Pharmacology and Toxicology Section, University of Florence, 6-50139 Florence, Italy*
Lorenzo Di Cesare Mannelli − *Department of Neuroscience, Psychology, Drug Research and Child Health, Neurofarba, Pharmacology and Toxicology Section, University of Florence, 6-50139 Florence, Italy*
Marta Ferraroni − *Department of Chemistry "Ugo Schiff" (DICUS), University of Florence, I-50019 Florence, Italy;* orcid.org/0000-0001-7258-738X
Maria Locuoco − *Department of Chemistry "Ugo Schiff" (DICUS), University of Florence, I-50019 Florence, Italy*
Antonella Capperucci − *Department of Chemistry "Ugo Schiff" (DICUS), University of Florence, I-50019 Florence, Italy;* orcid.org/0000-0003-1087-8143
Claudiu T. Supuran − *NEUROFARBA Department, Sezione di Scienze Farmaceutiche, University of Florence, 50019 Florence, Italy;* orcid.org/0000-0003-4262-0323

Complete contact information is available at: https://pubs.acs.org/10.1021/acsmedchemlett.4c00284

Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CA, carbonic anhydrase; NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; ROS, reactive oxygen species

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