Carboranyl Analogues of Ketoprofen with Cytostatic Activity against Human Melanoma and Colon Cancer Cell Lines

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*Supporting Information

ABSTRACT: Ketoprofen is a widely used nonsteroidal anti-inflammatory drug (NSAID) that also exhibits cytotoxic activity against various cancers. This makes ketoprofen an attractive structural lead for the development of new NSAIDs and cytotoxic agents. Recently, the incorporation of carboranes as phenyl mimetics in structures of established drugs has emerged as an attractive strategy in drug design. Herein, we report the synthesis and evaluation of four novel carborane-containing derivatives of ketoprofen, two of which are prodrug esters with an nitric oxide-releasing moiety. One of these prodrug esters exhibited high cytostatic activity against melanoma and colon cancer cell lines. The most pronounced activity was found in cell lines that are sensitive to oxidative stress, which was apparently induced by the ketoprofen analogue.

1. INTRODUCTION
Ketoprofen (Figure 1) is a nonsteroidal anti-inflammatory drug (NSAID) belonging to the group of substituted propionic acids. It is used to treat rheumatoid arthritis, osteoarthritis, dysmenorrhea, and to alleviate moderate pain. Ketoprofen has pharmacologic activity similar to that of other NSAIDs, and this is associated with the inhibition of prostaglandin synthesis catalyzed by both cyclooxygenase (COX) isoforms, COX-1 and COX-2. Its anti-inflammatory effects result from the inhibition of both COX-1 and COX-2, the latter being responsible for the production of prostaglandins under inflammatory conditions. In addition to its effects on COX, ketoprofen inhibits the lipoxygenase pathway of the arachidonic acid cascade. It is known that lipoxygenase inhibitors have also the potential to attenuate inflammation, and this inhibition is complementary to the COX inhibitory action. Furthermore, it is a powerful inhibitor of bradykinin, an important peptidic mediator of pain and inflammation.

Generally, the side effects of ketoprofen are similar to those of other classical NSAIDs. Serious side effects result mostly from gastrointestinal (GI) damage. In fact, ketoprofen is known to be one of the most ulcerogenic NSAIDs with a risk factor for serious GI complications close to that of ibuprofen. These side effects are mainly caused by the fact that ketoprofen is a nonselective COX inhibitor. In contrast to COX-2, COX-1 is constitutively expressed within the body and is responsible for the production of prostaglandins that have important physiological functions, and thus inhibition of this isoform can result in side effects.

The search for therapeutics with fewer side effects has led to the development of nitric oxide (NO)-releasing prodrugs of established drugs. Given the gastrototoxicity of ketoprofen, the design of an NO-releasing prodrug is beneficial as NO exhibits cytoprotective properties in the digestive mucosa. There are known examples of NO-releasing analogues of ketoprofen that have an aliphatic-linked NO-releasing moiety, i.e., a nitrate. The antinociceptive effect and efficacy of these compounds were tested on rats, and one of the compounds,

Figure 1. Ketoprofen and the nitric oxide (NO)-releasing analogue of ketoprofen HCT-2037.

Received: February 13, 2019
Accepted: April 17, 2019
Published: May 23, 2019

DOI: 10.1021/acsomega.9b00412
ACS Omega 2019, 4, 8824−8833
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Scheme 1. Synthesis of Ketoprofen Analogues 3 (from ortho-Carborane) and 4 (from meta-Carborane)

\[ \text{Scheme 1. Synthesis of Ketoprofen Analogues 3 (from ortho-Carborane) and 4 (from meta-Carborane)} \]

\[ \text{Diagram of Scheme 1 showing the synthesis of ketoprofen analogues 3 and 4 from ortho- and meta-carborane.} \]

2. RESULTS AND DISCUSSION

2.1. Molecular Design and Synthesis of Ketoprofen Analogues. Ketoprofen’s molecular structure features two phenyl rings, which, in principle, allow for introduction of the carborane moiety. In this work, the nonsubstituted ring was chosen to be replaced with a carborane cluster (Scheme 1, compounds 3 and 4) as metabolic transformations of ketoprofen occur at this phenyl ring.33 This is also a good surrogate for comparing the effect of a carborane substitution of a keto-bound benzene ring to the biological activity of the native drug.

As the most viable option for the synthesis of the carboranyl derivative of ketoprofen, the synthetic methodology involving a reaction between a lithio carborane and an acyl halide was chosen. This synthetic approach was performed with both an ortho-carborane and a meta-carborane to examine the effect of the position of the cluster carbon atoms on the chemical and biological properties of the compound. The meta isomer may be preferred as it is more stable toward deboronation compared to its ortho counterpart.23 On the other hand, the ortho cluster can be easily transformed into a nido-carborane,34 which may also be beneficial for the properties of the ketoprofen analogue.

The reaction conditions were chosen based on those previously reported by Zakharkin et al. for the synthesis of a methyl ketone derivative of ortho-carborane.35 To obtain a high yield of the monosubstituted derivative, a mixture of toluene and diethyl ether was used as the solvent for the ligation step, and an equivolumar amount of n-BuLi was reacted with ortho- or meta-carborane. The lithiated carborane was then added to freshly prepared 3-(1-cyanoethyl)benzoyl chloride dissolved in diethyl ether (Scheme 1). The addition step should be performed by adding the nucleophile to the electrophile to minimize the possibility of reduction of the newly formed keto group to a tertiary alcohol. By this approach, compounds 1 and 2 were obtained. Besides the characterization of the intermediates, the molecular structure of the ortho derivative 1 could be verified by X-ray
crystallography (Supporting Information (SI), Table S1, Figure S1).

The synthesis of the propionitrile derivatives 1 and 2 created intermediates that could be further transformed into the desired propionic acid derivatives 3 and 4 (Scheme 1). This is easily achievable by hydrolysis of the cyano group to a carboxylic group under acidic or basic conditions. To minimize the possibility of a deboronation side reaction of ortho-carborane, which is even more likely to occur due to the electron-withdrawing carbonyl group attached at the carborane cluster, the nitrile hydrolysis was done under acidic conditions. The hydrolysis was performed under reflux employing a mixture of acetic acid as the solvent and concentrated hydrochloric acid. This afforded the ketoprofen analogues 3 and 4 in quantitative yields. 3 and 4 were fully characterized and their structures were confirmed by X-ray crystallography (Figure 2; SI, Tables S2, S3 and Figures S2, S3).

Figure 2. Crystal structures of the ortho-carboranyl derivative of ketoprofen 3 (left) and the meta-carboranyl derivative 4 (right). (Oak Ridge thermal ellipsoid plot: displacement thermal ellipsoids are drawn at 50% probability level. Hydrogen atoms are omitted for clarity.) Detailed crystallographic parameters can be found in the SI (Tables S2 and S3).

To expand the functionality of the synthesized carboranyl derivatives of ketoprofen, a nitrate group was introduced to generate a prodrug with an NO-releasing moiety. Within the carborane-based ketoprofen analogues, the carboxylic group is the most accessible functionality to insert a linker with one or more nitrate groups attached. For this work, an aliphatic derivative of 1,3-dinitroglycerol was chosen because aromatic linkers are known to be associated with increased carcinogenicity. Furthermore, this allows the release of two NO molecules per inhibitor molecule. Activation of the carboxylic group as acyl halide and reaction with 1,3-dinitroglycerol or the corresponding alcoholate did not yield the desired product, even after prolonged reaction times and at elevated temperature. This may be attributed to the poor nucleophilicity of 1,3-dinitroglycerol. Finally, the dinitrate esters 5 and 6 could successfully be generated by employing Steglich esterification conditions with dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as coupling reagents (Scheme 2). Due to the presence of a chiral center in the nitrate esters 5 and 6, the methylene protons of the isopropyl group are diastereotopic and can be observed as pairs of doublets via coupling to the methine proton in the 1H NMR spectra.

2.2. Evaluation of COX Inhibitory and Cytotoxic Activities. All compounds were tested for their COX inhibitory activity employing a commercial enzymatic COX assay (“COX Fluorescent Inhibitor Screening Assay Kit”, Item no. 700100, Cayman Chemical, Ann Arbor, MI) as described elsewhere. However, only compound 5, the ketoprofen analogue bearing an ortho-carborane and a nitrate moiety, exhibited weak COX inhibition without selectivity for one of the isoforms (SI, Table S4). The other compounds did not exhibit any COX inhibitory activity.

To investigate the antitumor activity of the carboranyl analogues of ketoprofen, three melanoma as well as three colon cancer cell lines were used. The selection of the cell lines was based on their ability to express COX-2; A375, B16F10, B16, CT26CL25 are expressing COX-2, whereas HCT1116 and SW480 do not. The four synthesized compounds were applied at 0.8–50 μM, and the percentage of viable cells was determined by measuring mitochondrial respiration [MTT assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as well as DNA/RNA content of attached cells (crystal violet (CV) assay employing crystal violet). After 48 h incubation, the colorimetric assays revealed that only compound 6 affected cell viability of the tested cells in a dose-dependent manner with an inhibitory activity varying among the cell lines (Table 1).

Interestingly, the highest sensitivity was found for the COX-2-expressing melanoma cell line A375 and the COX-2-negative colon cancer cell line HCT1116, indicating that the cytotoxic activity of 6 is probably COX-2 independent. This is consistent with the observation that 6 does not exhibit COX inhibitory activity. Comparing the sensitivities among the different cell lines showed that the redox-sensitive cell lines are most susceptible to 6. The IC_{50} values determined with both assays

Scheme 2. Steglich Esterification To Generate the NO-Releasing Prodrugs 5 and 6

(i) With either 3 (for 5) or 4 (for 6) as starting material; DCC, DMAP, CH2Cl2.
(MTT, CV) were similar and showed that 6 affects the cell viability in a micromolar range; in contrast, ketoprofen did not affect cell viability in the same dose range (Figure 3). This result is in agreement with the previously reported low effectiveness of ketoprofen against different cell lines.45−47 However, a significant cytostatic effect was observed upon exposure of human cervical carcinoma (HeLa) and human colon cancer cells (Caco-2) to ketoprofen, which may be attributed to the NF-κB inhibition that was detected.16

Comparison of sensitivities toward 6 of malignant cells (Table 1) versus primary macrophages (IC$_{50}$ = 46.55 ± 3.36 μM) showed that the compound is relatively selective for cancer cells; a selectivity index toward the transformed phenotype is given in Table S5, SI. Given that 6 showed high activity against the A375 melanoma cells, this cell line was used to determine the mechanism of the cytotoxic effect of ketoprofen analogue 6. To determine the main cause of decreased cell viability, the influence of 6 on cell division and cell death was investigated. As presented in Figure 4A, cell division was inhibited upon 48 h treatment of A375 cells with 6. Within the same period, only 5% of double-positive, late-apoptotic cells were determined suggesting a negligible contribution of that type of cell death to decreased viability in response to compound 6 (Figure 4B). Accordingly, only a small amplification of total caspase activity was observed after the treatment (Figure 4C). Given that minor apoptosis usually correlates with autophagic processes, the cells were examined for the presence of autophagosomes. Indeed, enhanced autophagy was detected by flow cytometry after acridine orange (AO) staining in response to the treatment with 6 (Figure 4D). In fact, it is well documented that apoptosis and autophagy confront each other, but under some circumstances, autophagy becomes a regular way of cell death, so it can contribute to the cytotoxicity of compound 6.44

Table 1. Cytotoxic Activities of Compounds 3–6

| IC$_{50}$ (μM) assay | compounds | MTT | CV | MTT | CV | MTT | CV | MTT | CV |
|-------------------|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| cell line | | | | | | | | | |
| A375 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 17.15 ± 1.55 | 19.7 ± 0.0$^c$ |
| B16 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 23.1 ± 0.0$^c$ | 24.5 ± 0.0$^c$ |
| B16F10 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 47.25 ± 2.75 | 46.15 ± 3.86 |
| HCT116 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 11 ± 1.2 | 18.95 ± 0.95 |
| CT26CL25 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 32.55 ± 3.17 | 50 ± 0.0$^c$ |
| SW480 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | 50 ± 0.0$^c$ | 49.4 ± 0.6 |

$^a$IC$_{50}$ values determined by MTT and CV assays (three independent experiments each). $^b$A375: human melanoma, B16: mouse solid melanoma, B16F10: mouse metastatic melanoma, HCT116: human colon carcinoma, CT25CL26: mouse colon carcinoma, SW480: human colon carcinoma. $^c$Standard deviation varies in the third decimal place.

Figure 3. Effect of ketoprofen and 6 on the cell viability of different cell lines. Cells were exposed to 6 (A) or ketoprofen (B) for 48 h, and cell viability was analyzed using MTT (left panel) and CV assays (right panel). *p < 0.05 compared to untreated controls.
It has been reported that ketoprofen induced oxidative damage of gastrointestinal mucosa, and it was attempted to circumvent this damage by incorporating an NO-releasing moiety based on the cytoprotective, prostaglandin-like properties of NO. Since is designed to be an NO-releasing ketoprofen analogue, the level of intracellular as well as extracellular NO in response to the treatment was determined. The release of NO from a nitrate moiety is a three-electron reduction that is normally catalyzed by multiple possible enzymes. However, compound 6 was found not to affect the intracellular amount of NO in A375 cells (Figure 5A). Having in mind that A375 cells are able to produce NO, it can be concluded that compound 6 neither liberated NO nor changed endogenous production of this molecule in the melanoma cell line. Given that NO donors also release NO extracellularly and that the absence of intracellular NO release is not a proof that a compound is not able to liberate NO at all, spontaneous as well as the extracellular release of NO from 6 was measured. Release of NO in the extracellular compartment is typically determined by the Griess reaction that measures the resultant nitrite accumulation. Nitrite accumulation was measured in culture medium, conditioned culture medium (medium taken from cell cultures upon incubation for 72 h) that contains soluble cellular products or cell debris, as well as in the supernatants from A375 cells that were exposed to 6 (10–40 μM) for 48 h. However, the determined nitrite accumulation was at the detection limit of the Griess reaction (1.5 μM), even at the highest applied dose, suggesting that 6 did not release NO in the extracellular compartment. Moreover, the production of reactive oxygen species/reactive nitrogen species (ROS/RNS) was measured employing dihydrorhodamine-123 (DHR). Indeed, ROS/RNS production was elevated after the exposure to 6 (Figure 5B), indicating that the compound promoted oxidative stress, which...
correlates with the fact that cell lines highly susceptible to oxidative stress were affected most by 6.

3. CONCLUSIONS

We report the synthesis of two carboranyl analogues of ketoprofen bearing an ortho- or meta-carborane (3 and 4), as well as derivatives additionally bearing nitrate moieties (5 and 6). The ortho-carborane nitrate ester 5 showed weak COX inhibitory activity. On the other hand, compound 6, the meta-carborane analogue of 5, exhibited cytotoxic activity toward colon and melanoma cell lines in the micromolar range with a special effect on the A375 cell line. It was determined that the cytotoxic effect is due to its potential to promote autophagy and oxidative stress in cells. Further research should provide better insights into the mechanism of action and the molecular targets of the ketoprofen analogue 6.

4. EXPERIMENTAL SECTION

4.1. Syntheses. 4.1.1. Materials and Methods. All commercial reagents and solvents were used without further purification. Reactions including carboranes were carried out under a nitrogen atmosphere using the standard Schlenk technique. Compounds 2, 4, and 6 were synthesized with 10B-enriched m-carborane. For column chromatography, silica gel (60 Å) from the company ACROS was used. The particle size was in the range of 0.035–0.070 mm. Thin-layer chromatography was used to monitor the reaction process of the syntheses. For this purpose, glass plates coated with silica gel plates 60 F254 from the company MERCK were used. Carborane-containing substances were stained with a 5% solution of palladium chloride in methanol (MeOH). All received 1H, 13C, and 11B NMR spectra were recorded with an ABRUKER apparatus and were uncorrected. IR spectra were recorded with a BRUKER Daltonics APEX II FT-ICR spectrometer. For these measurements, dichloromethane, acetonitrile (ACN), methanol (MeOH), formic acid (FA), or a mixture of these solvents was used.

Compounds 1–6 were obtained and employed as racemic mixtures.

4.1.2. Synthesis of 2-[3-(1,2-Dicarba-closo-dodecaboranyloxy)phenyl]propionitride (2). This compound was synthesized in a manner similar as described for the ortho isomer, starting from 1.5 g (0.011 mol) of m-carborane. Compound 2 was obtained as a colorless solid. Yield: 32% (1.1 g, 3.65 mmol); mp: 80–81 °C. 1H NMR (CDCl3, 400 MHz): δ = 7.64–7.54 (m, 3H, CH aromat), 7.46 (t, 1H, CH aromat), 3.95 (q, JHH = 8 Hz, 1H, CHlute), 1.67 (d, JHH = 8 Hz, 3H, CHlute), 3.35–1.14 (m, 10H, BHlute), 12B{1H} NMR (CDCl3, 128 MHz): δ = −5.5 (br, 3B), −10.8 (br, 3B), −11.9 (s, 2B), −15.2 (s, 2B), 13C{1H} NMR (CDCl3, 100 MHz): δ = 188.4 (CO), 137.4 (qCH aromat), 131.7 (qCH aromat), 130.6 (CH aromat), 129.0 (CH aromat), 128.3 (CH aromat), 126.9 (CH aromat), 120.8 (CN), 55.0 (CH lute), 31.0 (CH), 21.2 (CH3); IR (KBr, cm−1): ν̃ = 3072 (m, ν(C-Hlute)), 2998–2870 (w, ν(C-Hlute)), 2615 (s, ν(C-Hlute)), 2247 (s, ν(C=N)), 1683 (s, ν(C=O)); HR-ESI-MS (positive, ACN) m/z [M + Na]+: calc for C12H18B10NO NaO: 302.2391, found: 302.2401; the observed isotopic pattern was in agreement with the calculated one. Elemental analysis: calc for C: 47.82, H: 6.47, N: 4.65, found for C: 47.79, H: 6.35, N: 4.49.

4.1.3. Synthesis of 2-[3-(1,2-Dicarba-closo-dodecaboranyloxy)phenyl]propionic Acid (3). Compound 1 (1 g, 3.32 mmol) was dissolved in 20 mL of acetic acid and 10 mL of 6 M HCl. The solution was left to reflux overnight and then it was neutralized with saturated NaHCO3. The resulting slurry was extracted with ethyl acetate (2 × 30 mL). The organic phase was collected and dried over anhydrous MgSO4, and the solvent was removed under reduced pressure to yield a yellow viscous oil. Purification was carried out by column chromatography (n-hexane/ethyl acetate 4:1). This gave compound 1 as a colorless solid. Yield: 32% (1.4 g, 4.6 mmol); mp: 69–70 °C. 1H NMR (CDCl3, 400 MHz): δ = 8.04 (d, 1H, CH aromat), 7.97 (s, 1H, CH aromat), 7.56 (t, 1H, CH aromat), 4.56 (s, 1H, CHlute), 4.00 (q, JHH = 8 Hz, 1H, CHL), 1.71 (d, JHH = 8 Hz, 3H, CHL), 3.2–1.7 (m, 10H, BHlute); 13B{1H} NMR (CDCl3, 128 MHz): δ = −1.3 (s, 1B), −2.7 (s, 2B), −8.0 (s, 3B), −11.7 (s, 2B), −13.4 (s, 2B); 13C{1H} NMR (CDCl3, 100 MHz): δ = 219.7 (CO), 185.2 (CN), 137.9 (qCH aromat), 134.0 (qCH aromat), 132.5 (CH aromat), 130.1 (CH aromat), 129.5 (CH aromat), 128.6 (CH aromat), 59.2 (CH lute), 32.1 (CH), 21.2 (CH3); IR (KBr, cm−1): ν̃ = 3044 (m, ν(C-Hlute)), 2992–2877 (w, ν(C-Hlute)), 2240 (w, ν(C=O)), 1687 (s, ν(C=O)); high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (negative mode, ACN) m/z [M − H]+: calc for C12H14B2O3NO: 209.2523, found: 209.2503; the observed isotopic pattern was in agreement with the calculated one. Elemental analysis: calc for C: 47.82, H: 6.47, N: 4.77, found for C: 47.79, H: 6.35, N: 4.67.

4.1.4. Synthesis of 2-[3-(1,2-Dicarba-closo-dodecaboranyloxy)phenyl]propanoic Acid (3). Compound 1 (1 g, 3.32 mmol) was dissolved in 20 mL of acetic acid and 10 mL of 6 M HCl. The solution was left to reflux overnight and then it was neutralized with saturated NaHCO3. The resulting slurry was extracted with ethyl acetate (2 × 30 mL). The organic phase was collected and dried over anhydrous MgSO4, and the solvent was removed under reduced pressure to yield a yellow viscous oil. Purification was carried out by column chromatography (n-hexane/ethyl acetate 2:1). Yield: 96% (1.02 g, 3.18 mmol); mp: 91–92 °C. 1H NMR (CDCl3, 400 MHz): δ = 7.99 (s, 1H, CH aromat), 7.96 (d, 1H, CH aromat), 7.62 (d, 1H, CH aromat), 7.46 (t, 1H, CH aromat), 4.55 (s, 1H, CH lute), 3.83 (q, JHH = 7.1 Hz, 1H, CHL), 1.57 (d, JHH = 7.1 Hz, 3H, CHL), 3.45–1.6 (m, 10H, BH lute); 13B{1H} NMR (CDCl3, 128 MHz): δ = −1.4 (s, 1B), −2.5 (s, 2B), −8.1 (s, 2B), −11.5 (s, 3B), −13.4 (s, 2B); 13C{1H} NMR (CDCl3, 100 MHz): δ = 185.2 (CO), 178.4 (COOH), 140.4 (qCH aromat), 133.7 (CH aromat), 133.4 (qCH aromat), 129.7 (CH aromat), 128.9 (CH aromat), 59.2 (CH lute), 44.9 (CH), 18.1 (CH3); IR (KBr, cm−1): ν̃ = 3088 (m, ν(C−...
H\textsubscript{cluster})), 3000–2726 (w, ν(C–H\textsubscript{aliph})), 2592 (s, ν(H–H)), 1713 (s, ν(C=O)), 1671 (s, ν(C=O)), 1263 (s, ν(C=O)), 1233 (s, ν(C=O)); HR-ESI-MS (negative mode, ACN) m/z [M – CO\textsubscript{2} – H]: calc for C\textsubscript{11}H\textsubscript{19}B\textsubscript{10}O\textsubscript{3}: 311.2628, found: 311.2672; the observed isotopic pattern was in agreement with the calculated one. Elemental analysis: calc for C: 37.19, H: 5.78, found for C: 37.37, H: 4.96, N: 5.80.

### 4.1.7. Synthesis of 1,3-Bis(nitrooxy)propan-2-yl-2-[(1,7-dicarba-closo-dodecaboranoyl)phenyl]propanoic Acid (6)

This compound was synthesized in a manner similar as described for the ortho isomer starting from 0.7 g (2.22 mmol) of 4. Compound 6 was obtained as a viscous clear and colorless oil. Yield: 92% (0.99 g, 2.0 mol). \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz): δ = 7.57 (d, 1H, CH\textsubscript{atromat}), 7.54 (s, 1H, CH\textsubscript{artomatic}), 7.45 (d, 1H, CH\textsubscript{atromat}), 7.39 (t, 1H, CH\textsubscript{aromatic}), 5.37 (m, 1H, CH), 4.75 (dd, J\textsubscript{HH} = 5.9 Hz, J\textsubscript{HH} = 12.7 Hz, 1H, CH), 4.63 (dd, J\textsubscript{HH} = 5.9 Hz, J\textsubscript{HH} = 12.7 Hz, 1H, CH), 4.55 (dd, J\textsubscript{HH} = 5.9 Hz, J\textsubscript{HH} = 12.7 Hz, 1H, CH), 4.47 (dd, J\textsubscript{HH} = 5.9 Hz, J\textsubscript{HH} = 12.7 Hz, 1H, CH), 3.79 (q, J\textsubscript{HH} = 7 Hz, 1H, CH), 3.08 (s, 3H, CH\textsubscript{cluster}), 1.54 (d, J\textsubscript{HH} = 4 Hz, 1H, CH), 3.36–1.49 (m, 10H, BH\textsubscript{cluster}); \textsuperscript{11}B\{\textsuperscript{1}H\} NMR (CDCl\textsubscript{3}, 128 MHz): δ = −10.8 (br, 4B), −12.9 (s, 3B), −15.2 (s, 3B); \textsuperscript{13}C\{\textsuperscript{1}H\} NMR (CDCl\textsubscript{3}, ppm): δ = 167.1 (CO), 167.8 (CO), 134.3 (qCH\textsubscript{aromatic}), 127.9 (qCH\textsubscript{aromatic}), 127.8 (qCH\textsubscript{aromatic}), 148.1 (CH\textsubscript{aromatic}), 182.8 (CH\textsubscript{aromatic}), 181.2 (CH\textsubscript{aromatic}), 131.4 (CH\textsubscript{aromatic}), 128.6 (CH\textsubscript{aromatic}), 127.9 (CH\textsubscript{aromatic}), 127.8 (CH\textsubscript{aromatic}), 69.4 (CH\textsubscript{2}), 69.2 (CH\textsubscript{2}), 66.9 (CH\textsubscript{2}), 55.1 (CH\textsubscript{aromatic}), 45.0 (CH), 181.1 (CH\textsubscript{3}); IR (KBr, cm\textsuperscript{-1}): ν = 3067 (m, ν(C–H\textsubscript{cluster})), 2617 (s, ν(H–H\textsubscript{cluster})), 1746 (m, ν(C=O)), 1647 (s, ν(C=O) or ν(N=O)), 1277 (s, ν(C=O) or ν(N=O)), 851 (s, ν(N=O))–HR-ESI-MS (negative mode, ACN, FA) m/z [M + FA – H]: calculated for the formic acid (FA) adduct C\textsubscript{11}H\textsubscript{19}B\textsubscript{10}N\textsubscript{2}O\textsubscript{11}: 521.2752, found: 521.2695; the observed isotopic pattern was in agreement with the calculated one. Elemental analysis: calc for C: 37.81, H: 5.07, N: 5.87, found for C: 37.47, H: 5.06, N: 5.37.

### 4.2. Cell Viability Materials and Methods

4.2.1. Reagents and Cells. Reagents were for the most part obtained from Sigma (St. Louis, MO). Annexin V-FITC (AnnV) was obtained from Biotium (Hayward, CA), and apostat was from R&D (R&D Systems, Minneapolis, MN). The melanoma cell lines A375, B16 and its metastatic subclone B16F10 as well as the colon cancer cell lines HCT116, SW480, and CT26CL25 were cultivated in 10% fetal calf serum (FCS) Roswell Park Memorial Institute (RPMI)-1640 medium with 2 mM L-glutamine, 0.01% sodium pyruvate, and antibiotics at 37 °C in a humidified atmosphere with 5% CO\textsubscript{2}. Upon trypsinization, cells were seeded in a certain number: 2.5 × 10\textsuperscript{3} cells/well for 96-well plates and viability assessment and 2 × 10\textsuperscript{4} cells/well for 24-well plates and flow-cytometric analysis. C57BL/6 mice, obtained from the animal facility at The Institute for Biological Research “Sinisa Stankovic”, were sacrificed for isolation of peritoneal resident macrophages. For viability assessment, cells were isolated by peritoneal lavage, washed, counted, seeded in 96-well plates and incubated overnight before drug application. Before treatment, nonadherent cells were removed. Carboxane-based ketoprofen analogues were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C for a month. 10% FCS–RPMI-1640 working solutions were prepared before the experiment started. In each experiment, control cultures were exposed to equal amounts of DMSO.

4.2.1. Cell Viability Tests. For viability assessment, two of the most frequently used tests were employed: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) and crystal violet (CV). Cells were exposed to 3–6
in a wide range of doses for a certain time interval, and the number of viable cells was determined as described.\(^2\) Nontreated cells were arbitrarily set to 100%, and according to this, results were expressed as the percentage of control.

4.1.10. Annexin V-FITC/PI, Acridine Orange Staining, and Caspase Detection. A375 cells were treated with 6 for 48 h and, after trypsinization, stained with Annexin V-FITC (Ann)/propidium iodide (PI) or apoptast as proposed by the manufacturer. To evaluate the presence of acidic vesicles, markers of autophagy, in the cytoplasm of cells, after-treatment staining with a 10 μM AO solution for 15 min at 37 °C was done. At the end of incubation, cells were washed and finally resuspended in phosphate buffer solution (PBS). At the end of all staining procedures, cells were analyzed with CyFlow Space Partec using the PartecFloMax software.

4.1.11. Cell Staining with Carboxyfluorescein Succinimidyl Ester (CFSE). For cell division rate determination, cells were prestained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (1 μM) for 10 min at 37 °C. After dye removal, cells were exposed to an IC\(_{50}\) dose of 6 and 48 h later analyzed by CyFlow Space Partec using the PartecFloMax.

4.1.12. Measurement of Intracellular Nitric Oxide. 4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM, 5 μM) was used for quantification of the intracellular level of NO. After an incubation time of 48 h, the cells were incubated in phenol-red-free RPMI containing DAF-FM for 1 h at 37 °C, washed, and additionally incubated for 15 min in serum/phenol-red-free conditions to complete de-esterification of intracellular diacetate. Cells were resuspended in phosphate buffer solution (PBS) and analyzed as described.

4.1.13. Measurement of Intracellular Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). Dihydrorhodamine-123 (DHR) (1 μM) was used for detection of ROS/RNS production. To determine the influence of 6 on the redox status of A375 cells, they were stained with DHR dye for 20 min prior to exposure to an IC\(_{50}\) dose of 6. After 48 h, cells were collected, washed, and resuspended in PBS and analyzed with CyFlow Space Partec using PartecFloMax.

4.1.14. Nitrite Detection. For detection of NO release in the culture supernatant, nitrite accumulation, as an indicator of spontaneous release of nitric oxide, 6 was diluted in the culture medium or conditioned medium, collected from cell cultures after three days of incubation. Certain aliquots of culture supernatants were mixed with the same volume of the Griess reagent prepared from 0.1% naphthylenediamine dihydrochloride and 1% sulfanilamide in 5% H\(_2\)PO\(_4\) mixed in a 1:1 ratio. Plates were incubated for 10 min at room temperature, and the absorbance was measured at 570 nm using a microplate reader. The nitrite concentration was calculated according to a standard curve derived from a range of NaNO\(_2\) concentrations.

4.1.15. Statistics. The results were obtained in triplicate. IC\(_{50}\) concentrations were calculated from at least three independent experiments. The significance of the differences between various treatments was calculated by the analysis of variance, followed by the Student–Newman–Keuls test. A p value of less than 0.05 was considered significant in comparison to untreated control cells.

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**ASSOCIATED CONTENT**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00412.

**COX inhibition assay:** Measured COX-1 and COX-2 inhibition values for compounds 3–6; Selectivity index values of 6 in melanoma and colon cancer cell lines (PDF)

Crystallographic data and crystal structure of 1 (CCDC 1880967); 3 (CCDC 1880968); 4 (CCDC 1880969) (CIF)

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Support from the Deutscher Akademischer Austauschdienst (DAAD, doctoral fellowship for A.B.), the DFG (HE 1376/38-1), the Graduate School BuildMoNa (A.B., W.N.), and the Ministry of Education, Science and Technological Development of the Republic of Serbia (project No. 173013) is gratefully acknowledged. The authors acknowledge the expert technical assistance of Mareike Barth and Johanna Wodtke (HZDR) and Stefanie Märcker and Ines Rein (Leipzig University). We acknowledge support from the German Research Foundation (DFG) and Leipzig University within the program of Open Access Publishing.

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