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Carborane-Based Tebufelone Analogs and Their Biological Evaluation In Vitro

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The presence of inflammatory mediators in the tumor microenvironment, such as cytokines, growth factors or eicosanoids, indicate cancer-related inflammatory processes. Targeting these inflammatory mediators and related signal pathways may offer a rational strategy for the treatment of cancer. This study focuses on the incorporation of metabolically stable, sterically demanding, and hydrophobic dicarba-*closo*-dodecaboranes (carboranes) into dual cyclooxygenase-2 (COX-2)/5-lipoxygenase (5-LO) inhibitors that are key enzymes in the biosynthesis of eicosanoids. The di-*tert*-butylphenol derivative tebufelone represents a selective dual COX-2/5-LO inhibitor. The incorporation of *meta*- or *para*-carborane into the tebufelone scaffold resulted in eight carborane-based tebufelone analogs that show no COX inhibition but 5-LO inhibitory activity in vitro. Cell viability studies on HT29 colon adenocarcinoma cells revealed that the observed antiproliferative effect of the *para*-carborane analogs of tebufelone is enhanced by structural modifications that include chain elongation in combination with introduction of a methylene spacer resulting in higher anticancer activity compared to tebufelone. Hence, this strategy proved to be a promising approach to design potent 5-LO inhibitors with potential application as cytostatic agents.

Introduction

Chronic inflammation can increase the risk of developing human cancer, e.g., colon or oesophageal cancer,[1] as inflammatory mediators like eicosanoids promote the development of carcinogenesis by participating in complex signaling processes.[2,3] Eicosanoids, in particular prostaglandins (PGs) and leukotrienes (LTs), are pro-inflammatory lipid mediators.[4] They are derived from arachidonic acid (AA) via two main signaling pathways mediated by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO).[3,5,6] Cyclooxygenases (COX) exist in three different isoforms: COX-1, COX-2, and COX-3. They catalyze the rate-determining step in the biosynthesis of PGs, prostacyclin, and thromboxane.[7–9] COX-3, a splicing variant of COX-1, is present in the central nervous system (CNS).[10] COX-1 is constitutively expressed in most tissues and is commonly known to be responsible for housekeeping functions, such as the protection of gastric mucosa, the maintenance of renal perfusion or the regulation of platelet activity.[11] COX-2 can be induced by pro-inflammatory stimuli, such as cytokines or tumor promoters, and is found in macrophages, fibroblasts, and leukocytes. COX-2 is also upregulated during inflammatory diseases, e.g., rheumatoid arthritis, cardiovascular diseases, and diseases related to airway functions, like asthma.[5,9,12] Furthermore, COX-2 is able to promote carcinogenesis in different cancer types, including colon, pancreatic or lung cancer.[9,13,14] COX inhibitors can be divided into non-selective, non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors (COXIBs). Non-selective COX inhibition results in adverse gastrointestinal effects by blocking protective actions of COX-1 derived PGs.[8,11,13] As the binding pocket of COX-2 is approximately 25% larger due to the substitution of three key amino acids, the implementation of bulkier inhibitors enables COX-2 selective inhibition.[12,13] Selective COX-2 inhibitors, e.g., celecoxib, reveal lower gastrointestinal (GI) toxicity in general. However, they are nowadays used only in specific short-term indications or have been removed from the market due to their cardiovascular adverse effects caused by the reduction in endothelial prostaglandin I_2 (PGI_2) and increased levels of platelet aggregator thromboxane A_2 (TXA_2).[11,15]

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The non-heme, iron-containing oxidoreductase 5-LO belongs to a heterogeneous family of lipid peroxidizing enzymes catalyzing the conversion of AA to LTs with the help of 5-lipoxygenase-activating protein (FLAP).[5,16,17] 5-LO is involved in the regulation of the immune system and is mainly found in myeloid cells that originate from the bone marrow. 5-LO may be stimulated by growth factors and further pro-inflammatory stimuli, for example, cytokines.[18,19] Indeed, 5-LO upregulation and LT overproduction are related to hypersensitivity reactions, inflammatory diseases, and allergic disorders. It has also been observed in different types of epithelial cancers like breast, colon, lung, and prostate cancer.[19–21] Further, it has been related to inhibition of apoptosis in renal oesophageal and breast cancer.[17,22] LTs represent important paracrine lipid mediators and are involved in host defense, inflammatory processes, and cellular signaling, including apoptosis.[23] For instance, leukotriene B_4 (LTB_4) is involved in carcinogenesis by influencing tumor cell proliferation, differentiation, apoptosis as well as migration, invasion of carcinoma cells, and angiogenesis.[24] Lipoxygenase (LO) inhibitors can be divided into four different groups, namely redox (non-competitive), iron-chelating, non-redox (competitive) and allosteric inhibitors.[5,23,25] Besides having a short half-life, 5-LO inhibitors often exhibit hepatotoxicity due to the formed chemically reactive metabolites in liver.[23]

Considering the pro-inflammatory and carcinogenic effects of PGs and LTs, dual COX-2/5-LO inhibition represents a rational concept for the discovery of drugs showing enhanced anti-inflammatory profiles. Dual COX-2/5-LO inhibitors may prevent the upregulation of the respective opposed signaling pathway by blocking both targets.[26] Thus, balanced COX-2/5-LO inhibition may lower the risk for the appearance of severe adverse effects, such as GI injury and hypersensitive reactions.[5,6,11] Since PGs and LTs have complementing effects on pathogenesis of cancer and tumor progression, dual COX-2/5-LO inhibition may represent a more effective way to treat cancer and to prevent the pathogenesis of cancer.[14,19,20,27]

Tebufelone (NE-11740, 1-[3,5-bis(1,1-dimethylethyl)-4-hydroxy-phenyl]-5-hexyn-1-one, Figure 1) is a potent anti-inflammatory, analgesic, and antipyretic dual COX-2/5-LO inhibitor with enhanced safety profile relative to traditional NSAIDs.[28,29] It was originally designed by using information derived from structure-activity relationship (SAR) studies of anti-inflammatory AA analogs and selected antioxidants. Tebufelone showed anti-inflammatory activity on rat peritoneal macrophages and in human whole blood on a micromolar scale.[28–30] Moreover, it exhibited anti-inflammatory activity in various animal models, including rat carrageenan-induced paw edema or rat adjuvant-induced arthritis assay.[31] Janusz and co-workers further disclosed that 5-keto-substituted metabolites of tebufelone, namely dihydrodimethylbenzofuranes (DHDMBFs, Figure 1) lacking the anti-oxidant moiety, are active anti-inflammatory agents as well as potent COX-2/5-LO inhibitors with moderate selectivity for COX-2.[32–34] However, animal models indicated that repeated administration of tebufelone for more than three weeks resulted in significant hepatotoxicity.[35]

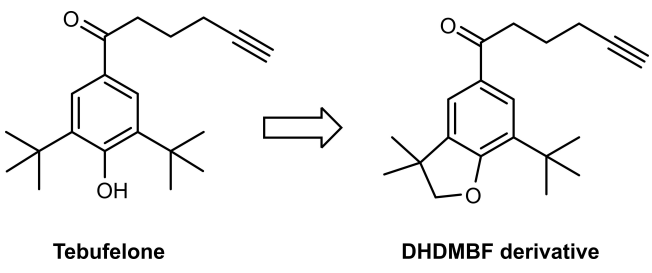


Figure 1. Dual COX-2/5-LO inhibitor tebufelone and its 5-keto-substituted 7-*tert*-buty1-2,3-dihydro-3,3-dimethylbenzofuran (DHDMBF) metabolite.

An emerging approach to increase metabolic stability of carbon-based drugs is the use of boron clusters, such as dicarba-*closo*-dodecaborane(12) ($C_2B_{10}H_{12}$, carboranes).[36–38] Carboranes are non-toxic, icosahedral boron clusters in which two BH^- vertices are replaced by two neutral CH groups.[39] Depending on the position of carbon atoms in the cluster, three different regioisomers, namely 1,2- (*ortho*), 1,7- (*meta*), or 1,12-dicarba-*closo*-dodecaborane (*para*), are reported.[36,40] In addition to their remarkable hydrophobicity, 3D-orthogonal functionalization of the carborane cluster enables to tune the pharmacokinetics of potential drug candidates.[38,40–43] Multiple non-covalent interactions, like dihydrogen bond formation, may increase the affinity to biological target, including enzymes or receptor proteins.[36,43,44] Due to a slightly larger van-der-Waals diameter compared to a phenyl ring (carborane: 5.25 Å, phenyl ring: 4.72 Å),[36,37,45] carboranes are frequently used as phenyl mimetics for biologically active compounds.[37,38,42,46]

Our group has intensively investigated the introduction of carborane moieties into NSAIDs and COXIBs in recent years.[47,48] For instance, the replacement of the chlorophenyl substituent of indomethacin by *nido*-carborane led to highly selective COX-2 inhibitors with inhibitory activity in the nanomolar range.[49] An approach to obtain selective and potent carborane-based 5-LO inhibitors as analogs of Rev-5901 resulted in decreased inhibitory activity toward 5-LO.[50] However, the quinoline-containing analog, CarbZDChin, showed increased cytotoxicity on colon carcinoma in vitro and in vivo.[51] We have recently reported the first carborane-based dual COX-2/5-LO inhibitors that are derived from RWJ-63556 showing excellent inhibitory potential toward COX-2 and 5-LO, accompanied by high anticancer activity on the A357 melanoma cell line.[52]

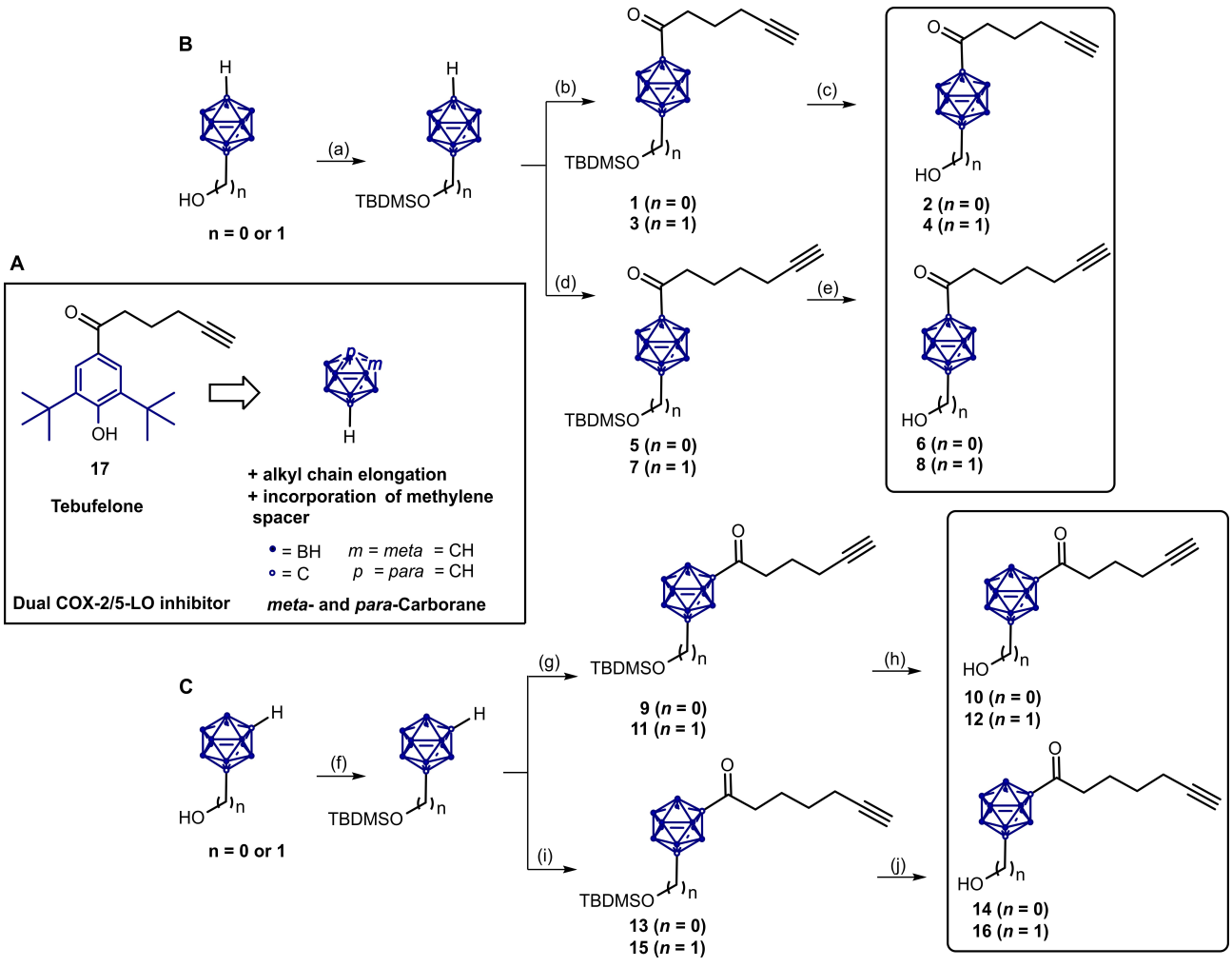
Herein, we report the synthesis of eight carborane-containing tebufelone analogs, their inhibitory potential toward COX-1, COX-2, and 5-LO, as well as their cytotoxicity on five human cancer cell lines.

Results and Discussion

Design and synthesis of tebufelone analogs

Our synthetic strategy is based on the bioisosteric replacement of unsubstituted or substituted phenyl rings that are found in typical carbon-based dual COX-2/5-LO inhibitors, by sterically demanding, metabolically stable carboranes. Tebufelone ac-

commodates a *para*-substituted phenol moiety and two *tert*-butyl groups that are arranged in *ortho*-position to the hydroxyl group. In this work, the bulky di-*tert*-butylphenyl moiety of tebufelone was replaced by carborane moieties. As *ortho*-carborane is prone to deboronation under physiological conditions, the stable *meta*- and *para*-carboranes were chosen. Moreover, the length of the alkyl chain of the keto substituent and the hydroxyalkyl substituent was extended, as the comparison of IC_{50} values of methyl to pentyl substituents at the 5-position of DHDMBFs revealed that an increase in chain length enhanced COX-2 selectivity.[33] While carborane analogs **2** and **10** (Scheme 1) were designed by incorporating *meta*- and *para*-carboranes, compounds **4**, **6**, **8**, **12**, **14**, and **16** were modified by elongation of the alkyl chain of the keto substituent (Scheme 1, compounds **6** and **14**) and by incorporation of a methylene spacer between the oxygen atom of the hydroxyl group and the carbon atoms of the corresponding carboranes (Scheme 1, compounds **4**, **8**, **12**, and **16**). 1-Hydroxy-*meta*-carborane,[53] 1-(hydroxymethyl)-*meta*-carborane,[54] 1-hydroxy-*para*-carborane,[53] and 1-(hydroxymethyl)-*para*-carborane[54] were prepared according to published procedures. Afterwards, all alcohols were quantitatively converted to the corresponding *tert*-butyldimethylsilyl (TBDMS) ethers by conventional silylation reactions with excess *tert*-butyldimethylsilyl chloride (TBDMSCl) in the presence of imidazole or NEt_3 catalyzed by 4-dimethylaminopyridine (DMAP). Ketones **1**, **3**, **5**, **7**, **9**, **11**, **13**, and **15** were obtained by monolithiation with *n*BuLi followed by reaction with one equivalent of the corresponding methyl ester in moderate yields (28–39%). Monolithiation is challenging and is depending on reaction temperature, time, and solvent. However, reaction kinetics of S_N-type reactions are also depending on the electrophile. The low activity of the carbonyl carbon atom of the methyl ester results in moderate conversion of the TBDMS-protected carboranyl alcohols to the desired ketones.



Scheme 1. A. General strategy for modification of tebufelone. **B.** Synthesis of *para*-carborane-based tebufelone analogs **2**, **4**, **6**, and **8**. Reagents and conditions: (a) 1. TBDMSCl, imidazole or NEt_3 and DMAP, CH_2Cl_2, 0 °C→RT, 48 h; 2. $HCl_{(aq)}$, RT; (b) 1. *n*BuLi, Et_2O, 0 °C→RT, 2 h; 2. methyl 5-hexynoate, 0 °C→RT, 24 h; 3. $HCl_{(aq)}$, RT; (c) 1. TBAF, THF, 0 °C, 20–30 min; 2. H_2O, RT (d) 1. *n*BuLi, Et_2O, 0 °C→RT, 2 h; 2. methyl 6-heptynoate, 0 °C→RT, 24 h; 3. $HCl_{(aq)}$, RT; (e) 1. TBAF or $HCl_{(aq)}$, THF, 0 °C, 20 min or 24 h; 2. H_2O, RT; **C.** Synthesis of *meta*-carborane-based tebufelone analogs **10**, **12**, **14**, and **16**. Reagents and conditions: (f) 1. TBDMSCl, imidazole or NEt_3 and DMAP, CH_2Cl_2, 0 °C→RT, 48 h; 2. $HCl_{(aq)}$, RT; (g) 1. *n*BuLi, Et_2O, 0 °C→RT, 2 h; 2. methyl 5-hexynoate, 0 °C→RT, 24 h; 3. $HCl_{(aq)}$, RT; (h) 1. TBAF, THF, 0 °C, 20–30 min; 2. H_2O, RT; (i) 1. *n*BuLi, Et_2O, 0 °C→RT, 2 h; 2. methyl 6-heptynoate, 0 °C→RT, 24 h; 3. $HCl_{(aq)}$, RT; (j) 1. TBAF or $HCl_{(aq)}$, THF, 0 °C, 20 min or 24 h; 2. H_2O, RT.

The formation of the ketones was always accompanied by the recovery of starting material. Finally, the carborane-based tebufelone analogs **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** were obtained by deprotection of the corresponding silyl ethers in the presence of tetra-*n*-butylammonium fluoride (TBAF) in excellent yields (91–98%, Scheme 1). All compounds were fully characterized by NMR and IR spectroscopy, mass spectrometry, and elemental analysis. Molecular structures of compounds **2**, **6**, and **8** were determined by single crystal X-ray crystallography (for further details see Supporting Information). Deposition numbers 2256475 (for **2**), 2256476 (for **6**), and 2256477 (for **8**) contain the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service.

Solubility and chemical stability in organic solvents, like dimethyl sulfoxide (DMSO), are crucial for biological investigations. The stability of the tebufelone analogs **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** in aqueous DMSO-d_6 in air at room temperature was studied by ^{1}H- and ^{11}B{^{1}H}-NMR spectroscopy for four weeks, confirming that all compounds are stable (see Supporting Information, Figures S1–S2).

Evaluation of inhibitory potential toward COX and 5-LO

The carborane-containing tebufelone analogs **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** as well as tebufelone were tested in vitro for their inhibitory potential toward ovine COX-1 and human recombinant COX-2 using the COX Fluorescent Inhibitor Screening Assay Kit (Cayman Chemical Company). Herein, the selective COX-2 inhibitor celecoxib and the COX-1 inhibitor SC-560 served as references. The carborane-containing tebufelone analogs showed no or only very low inhibitory activity towards both COX-1 and COX-2 at a concentration of 100 μM (see Supporting Information, Table S1) rendering them as inactive. In comparison, tebufelone inhibited 65% of COX-2 and 40% of COX-1 activity at this concentration indicating an IC_{50} value in the higher micromolar range in this assay. Tebufelone was previously demonstrated to inhibit cyclooxygenases, however with IC_{50} values in the higher nanomolar[32] to lower micromolar range[29] as determined by an enzyme and a radioimmunoassay. The weaker inhibition profile observed herein might be related to the higher substrate concentrations of 100 μM arachidonic acid applied in our experiments compared to the previously reported methods; however, exact substrate-concentration dependent IC_{50} values were not further determined due to the lack of activity of the tested compounds.

To evaluate the 5-LO inhibitory potential, compounds **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** were tested in an intact cell assay using polymorphonuclear leukocytes (PMNL) and tebufelone as reference. All compounds showed IC_{50} values below 1 μM (Table 1), with only moderate loss of potency compared to the reference tebufelone (**17**). Therefore, the incorporation of the carborane moiety seems to be well tolerated leading to strong inhibitors of 5-LO product formation.

Table 1. IC_{50} values for inhibition of 5-LO product formation of tebufelone (**17**) and its carborane derivatives **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** in intact PMNL. Data are presented as mean of at least three independent experiments (see Supporting Information, Figure S3 and (for 95% CIs), Table S2).

Compound	IC_{50} [μM]	Compound	IC_{50} [μM]
2	0.50	**10**	0.76
4	0.59	**12**	0.42
6	0.37	**14**	0.64
8	0.33	**16**	0.33
17	0.22		

In vitro evaluation of cytotoxicity on human cancer cell lines

NSAIDs are effective in the reduction of pro-inflammatory metabolites of arachidonic acid, but they manifest gastrointestinal and cardiovascular adverse effects.[55] Tebufelone as a dual COX-2/5-LO inhibitor exhibits higher selectivity for COX-2 compared to COX-1.[33] There are reports on tebufelone as an anti-inflammatory drug tested in vitro on rat peritoneal macrophages and human whole blood.[29,56] However, studies of the effect this drug has on cancer cells are deficient. This work is demonstrating for the first time the activity of tebufelone and its carborane analogs on cancer cells.

The anticancer activity of compounds **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** was screened on different human carcinoma cell lines. Based on COX-2 and 5-LO expression, the melanoma cell line A375, three colon cancer cell lines HT29, SW480, and SW620, as well as a lung cancer cell line A549 were selected.[26,52,57] Tebufelone (**17**) was used as a reference compound. The influence of the incorporated carborane moieties on cancer cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) assays (Table 2, see Supporting Information, Table S3).

Cancer cells were treated with compounds **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** in concentrations from 0 to 50 μM for 72 h. All treatments affected the cell viability in a dose-dependent manner. Results obtained from viability screening illustrated that the activity of the carborane-based tebufelone derivatives corresponded to the paternal compound **17** (Table 2, see Supporting Information, Figures S4 and S5). The IC_{50} values calculated from the MTT test were slightly lower than the IC_{50}

Table 2. IC_{50} values [μM] of tebufelone (**17**) and its carborane derivatives **2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16** in human carcinoma cell lines. Data are presented as mean ± SEM of three independent experiments for MTT test.

Cell line	A375	HT29	SW480	SW620	A549
Assay	MTT	MTT	MTT	MTT	MTT
2	34.3 ± 6.1	> 50	24.7 ± 0.2	> 50	42.3 ± 2.9
4	38.3 ± 1.7	24.8 ± 2.4	> 50	38.2 ± 1.9	41.2 ± 3.6
6	> 50	47.0 ± 1.8	31.5 ± 0.4	> 50	42.0 ± 0.4
8	27.1 ± 2.0	9.06 ± 0.8	32.0 ± 2.7	24.1 ± 3.2	18.6 ± 1.7
10	32.8 ± 6.1	44.6 ± 5.4	27.3 ± 3.3	41.2 ± 1.4	42.6 ± 2.1
12	25.1 ± 0.5	19.4 ± 1.8	36.0 ± 2.2	31.9 ± 0.8	29.9 ± 4.1
14	26.3 ± 4.3	39.4 ± 2.6	23.8 ± 3.3	34.2 ± 4.3	32.8 ± 3.1
16	24.3 ± 3.6	14.8 ± 1.8	33.6 ± 2.5	21.3 ± 1.7	20.0 ± 2.1
17	21.3 ± 1.8	18.8 ± 0.0	33.6 ± 2.7	37.9 ± 3.7	33.6 ± 4.6

values calculated from the CV test. This discrepancy can be ascribed to hindering cell proliferation, thereby increasing the quantity of CV dye internalized by the cells. Accordingly, the MTT test was found as more appropriate for the estimation of IC_{50} values.

The most potent compound in reducing the cell viability was derivative **8** that showed increased cytotoxicity for the COX-2- and 5-LO-dependent HT29 colon adenocarcinoma cell line (IC_{50} values were 9.06 μM in MTT and 15.3 μM in CV assay). Since this compound displayed lower IC_{50} values compared to the commercial inhibitor tebufelone, demonstrating a more potent antitumor effect, it was selected for further analyses of the drug's mode of action on HT29 cells.

Exposure of peritoneal exudate cells to the highest applied dose (200 μM) of **8** decreased cell viability by approx. 40% (see Supporting Information, Figure S5). Thus, the selectivity index calculated for HT29 cells would be higher than 22 (MTT test), indicating the outstanding selectivity of **8**.

Annexin V/PI (propidium iodide) staining detected the presence of early and late apoptotic cells in approximately 10% (Figure 2A). In parallel, the activation of key mediators of apoptotic stimuli, caspases, was not detected with a pan-caspase inhibitor (Figure 2B). Moreover, the acridine orange (AO) staining revealed that derivative **8** did not promote an autophagic process (Figure 2C). The main reason for viability decrease induced by **8** was obviously inhibition of proliferation, according to diacetate succinimidyl ester (CFSE) staining (Figure 2D). Taken together, a cytostatic rather than a cytotoxic effect of compound **8** was observed. Production of reactive oxygen species (ROS) and NO was measured by dihydrorhodamine (DHR) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF) staining, respectively. The production of ROS and NO was significantly affected by the treatment with **8** (Figures 2E and F), suggesting that H_2O_2, peroxynitrite, and NO could be responsible for the antiproliferative effect of this compound. Elevated ROS levels in tumor cells can lead to suppression of tumor cell proliferation.[58] When the levels of ROS are elevated beyond the toxic threshold, they can lead to cell death, apoptosis, and senescence.[59] Nitric oxide is another player with a dual role in tumor development and tumor suppression. In general, low levels of NO promote cell proliferation and anti/apoptotic response. High levels of NO can induce cell cycle arrest, apoptosis, and senescence through both oxidative and nitrosative stress.[60] Therefore, modulation of ROS/NO in cancer treatment is a very promising therapeutic strategy, with a limitation based on the high cellular and tumor microenvironmental specificity in handling and response to ROS.[61]

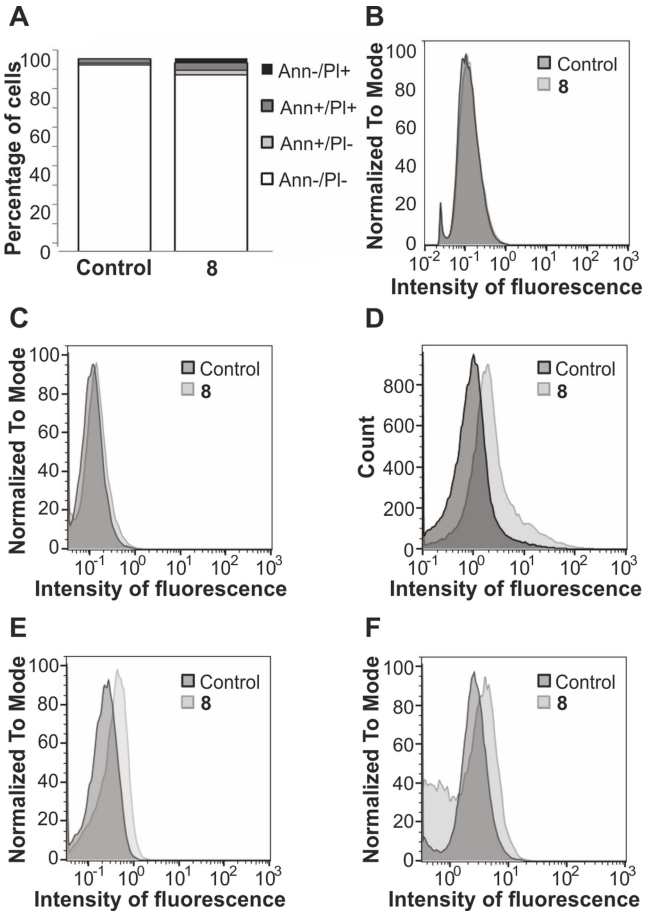


Figure 2. Compound **8** has a cytostatic effect on the colon cancer cell line. HT29 cells were treated with an IC_{50} dose of **8** for 72 h and stained with (A) Annexin V/PI, (B) Apostat, (C) AO, (D) CFSE, (E) 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF), and (F) dihydrorhodamine (DHR). Cells were analyzed by flow cytometry; one representative experiment out of three is shown.

Consequently, the observed cytotoxic effects of compound **8** might be based on oxidative stress that produces cell injury and death, rather than the inhibition of 5-LO. Additionally, there was no lipid accumulation detected in the cytoplasm of viable cells (data not shown), and overall, the cell morphology upon 72 h treatment with **8** was not remarkably changed (see Supporting Information, Figure S6). This work gives evidence that the carborane-based tebufelone derivatives have a different mode of action compared to recently presented carborane-based RWJ-63556 derivatives.[52]

Conclusions

Inflammation, in particular the presence of inflammatory mediators in the tumor microenvironment, can be regarded as one of the hallmarks of cancer. Both COX-2 and 5-LO are key enzymes catalyzing the conversion of AA to PGs and LTs that are involved in pro-inflammatory processes, including the development of cancer. In this study, we present the synthesis of eight carborane-based analogs (**2**, **4**, **6**, **8**, **10**, **12**, **14**, and **16**) of tebufelone, as well as their inhibitory and cytotoxic activity. The bioisosteric replacement of the di-*tert*-butylphenyl moiety by *meta*- or *para*-carborane and alkyl chain elongation was investigated. All carborane-based tebufelone analogs showed no or very low COX inhibitory activity but retained inhibitory potential toward 5-LO. The incorporation of a methylene spacer in-between the carbon atom of the carborane and the adjacent hydroxyl group enhances both 5-LO inhibitory and anticancer activity of the corresponding analogs **8**, **12**, and **16**. However,

no clear trend can be observed regarding the different influence of both carborane regioisomers on 5-LO inhibitory potential as well as on anticancer activity. Compound **8** represents the most potent carborane-based analog of tebufelone for human colon adenocarcinoma cells by exhibiting an antiproliferative effect. Interestingly, it further reveals excellent selectivity for tumor cells in addition to improved cytotoxicity. Subsequent elucidation of the mechanism indicates that the cytostatic activity of compound **8** may be independent from the initially targeted inhibition of COX-2 and 5-LO, but instead might be associated with the modulation of intracellular reactive oxygen species and NO resulting in oxidative stress and related cell death. Overall, carborane-based tebufelone analog **8** is a promising candidate for further assessment and detailed mechanistic and in vivo studies.

Experimental Section

Syntheses

Materials, methods, and procedures: See Supporting Information for further details.

Biological data

COX inhibition studies: The COX inhibition activity against ovine COX-1 and human COX-2 was determined using the fluorescence-based COX assay *COX Fluorescent Inhibitor Screening Assay Kit* (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions as previously reported by us.[48]

5-LO inhibitory studies in intact cells: For determination of 5-LO inhibitory activities in intact cells, freshly isolated PMNL (5×10^6) were re-suspended in phosphate buffered saline (PBS) (pH 7.4) containing 1 mg/mL glucose and 1 mM $CaCl_2$. After preincubation with **2**, **4**, **6**, **8**, **10**, **12**, **14**, **16**, and tebufelone (**17**) in DMSO for 15 min at 37 °C, 5-LO product formation was stimulated by addition of calcium ionophore A23187 (2.5 μM in MeOH) and exogenous arachidonic acid (20 μM in EtOH). After 10 min at 37 °C, the reaction was stopped by addition of ice-cold methanol (1 mL). HCl (30 μL, 1 M), prostaglandin B1 (200 ng), and PBS (500 μL) were added and the formed metabolites were extracted and analyzed by HPLC as described previously.[62] 5-LO product formation was determined as the amount of 5-LO products produced (nanograms) per 10^6 cells, which includes leukotriene B_4 (LTB_4), its all-*trans* isomers, and 5-H(P)ETE. Cysteinyl LTs C_4, D_4, and E_4 as well as oxidation products of LTB_4 were not detected. Data were normalized to vehicle control (DMSO), and either IC_{50} values and 95% confidence intervals (CIs) or means ± SEM of at least three independent measurements were calculated. The selective 5-LO inhibitor BWA4 C (0.1 μM) was used as control and inhibited 5-LO product formation by 89.9% ± 0.6.

Reagents and cells: Annexin V-FITC was obtained from Biotium (Hayward, USA), and Apostat from R&D (R&D Systems, Minneapolis, USA). PI, MTT, CV, and AO were purchased from Sigma (St Louis, USA), and CFSE was obtained from Abcam (Cambridge, UK). Melanoma cell line A375 and colon cancer cell lines HT29, SW480, SW620, and A549 were cultivated in 10% fetal calf serum (FCS) RPMI-1640 medium with 2 mM L-glutamine, 0.01% sodium pyruvate, and antibiotics at 37 °C in a humidified atmosphere with 5% CO_2. C57BL/6 mice, obtained from the animal facility at Institute for Biological Research "Siniša Stanković", were sacrificed for the isolation of peritoneal resident macrophages. The use of animals was in agreement with the rules of the European Union and the European Community guidelines (EEC Directive of 1986; 86/609/EEC). The protocol for isolation of cells was approved and allowed by the national licensing committee at the Department of Animal Welfare, Veterinary Directorate, Ministry of Agriculture, Forestry, and Water Management of the Republic of Serbia (Permission No. 323-07-02147/2023-05).

Cell viability assays: Viability assays were performed as described in Braun et al.[52] The cells were exposed to tested compounds in the range of 0 to 50 μM for 72 h. For the MTT assay, the cells were then incubated with 0.5 mg/mL MTT, the supernatant was removed, and 50 μL DMSO was added. For the CV assay, the cells were fixed in paraformaldehyde and stained with 2% CV in PBS. Then the cells were washed and the dye was dissolved in 33% acetic acid. For both assays, the absorbance at 540 nm and 670 nm was measured. The non-treated cells were arbitrarily set to 100% and the results were calculated as the percentage of the control.

Cell proliferation assay: To determine the cell division rate, cells were pre-stained with 1 μM CFSE dye for 10 min at 37 °C. After the dye removal, cells were exposed to an IC_{50} dose of **8** for 72 h. The flow cytometric analysis was done on CyFlow® Space (Partec, Muenster, Germany) and analyzed using FlowJo software.

Statistics: The IC_{50} concentrations were calculated from at least three independent experiments. The significance of the differences between various treatments was calculated by the t-test or the analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. A p-value less than 0.05 was considered significant.

Supporting Information

Additional references cited within the Supporting Information.[63–70]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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