Borcalein: a Carborane-Based Analogue of Baicalein with 12-Lipoxygenase-Independent Toxicity

Robert Kuhnert[+],[a] Lydia Kuhnert[+],[b] Menyhárt-B. Sárosi,[a] Sven George,[c] Dijana Draca,[d] Svetlana Paskas,[d] Bettina Hofmann,[c] Dieter Steinhilber,[c] Walther Honscha,[b] Sanja Mijatović,[d] Danijela Maksimović-Ivanić,[d] and Evamarie Hey-Hawkins*[a]

12-Lipoxygenase is crucial for tumour angiogenesis. 5,6,7-Trihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (baicalein) is a suitable inhibitor for this enzyme but is rapidly metabolised *in vivo*. Thus, an improvement of the metabolic stability is necessary to enhance the therapeutic efficiency. An emerging approach to enhance metabolic stability of carbon-based pharmaceuticals is the use of metabolically stable, non-toxic boron clusters, such as dicarba-*closo*-dodecaborane(12)s (carboranes) as phenyl mimetics. Therefore, the unsubstituted phenyl ring of baicalein was replaced by *meta*-carborane, resulting in borcalein, the carborane analogue of baicalein. This substitution resulted in a decreased inhibitory activity toward 12-lipoxygenase, but led to increased toxicity in melanoma (A375, B16, B16F10) and colon cancer cell lines (SW480, HCT116, CT26CL25) with decreased tumour selectivity in comparison to baicalein. Surprisingly, borcalein displays a different mechanism of cytotoxicity with increased intracellular production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and nitric oxide (NO).

Introduction

Baicalein is a herbal ingredient from the root of *Scutellaria baicalensis*, used in traditional Chinese medicine for the treatment of fever, pain and inflammation.[1] It inhibits the enzyme 12-lipoxygenase (12-LO) that is involved in the metabolism of arachidonic acid and the progression of cancer.[2,3] The activity of 12-LO *in vivo*, represented by the accumulation of its metabolite 12-hydroxyeicosatetraenoic acid (12(S)-HETE), serves as a marker of melanoma invasiveness[4] and metastatic potential.[5] Thus, inhibition of this enzyme was already shown to be important in preventing metastasis and angiogenesis.[6] Several studies verify that baicalein has a broad anti-tumour activity against breast cancer cells, hepatocellular carcinoma, leukaemia, colon, lung and prostate cancer cells.[7] Furthermore, baicalein has beneficial effects in the treatment of gliomas, but high therapeutic doses are needed.[8] Since baicalein is known to be rapidly metabolised *in vivo*,[9] an improvement of metabolic stability is necessary to enhance the therapeutic efficiency.

An emerging approach to overcome metabolic instability of carbon-based pharmaceuticals is the use of metabolically stable, non-toxic boron clusters, such as dicarba-*closo*-dodecaborane-(12)s ($C_2B_{10}H_{12}$, carboranes).[10,11] Carboranes are boron clusters in which two BH^- vertices are substituted by neutral CH groups; they are isosteric to cyclic functional groups, such as phenyl rings, pyridine or cyclopentyl rings.[12] Their hydrophobicity and the possibility for a three-dimensional functionalisation can facilitate intracellular enrichment and the affinity to hydrophobic enzymatic binding pockets.[12,13] Furthermore, an introduction of hydrophobic residues like carboranes into biologically active compounds is known to increase cell penetration by passive diffusion.[6]

Carboranes are frequently used as phenyl mimetics for biologically active compounds.[11] For example, the introduction of a carborane moiety into indomethacin increased the selectivity toward the cancer-specific isoform of cyclooxygenase, COX-2.[14] As shown previously,[13,15] also other members of the lipoxygenase family were efficiently inhibited by carborane-based compounds, e.g. 5-lipoxygenase (5-LO) by the carborane analogue of Rev-5901 (CarbORev-5901), leading to increased selective cytotoxicity toward aggressive melanoma and colon adenocarcinoma cell lines.

We herein report the effect of the replacement of the phenyl moiety in the 12-LO inhibitor baicalein by *meta*-carborane.

[a] *Dr. R. Kuhnert,[+] Dr. M.-B. Sárosi, Prof. Dr. E. Hey-Hawkins*
Institute of Inorganic Chemistry
Faculty of Chemistry and Mineralogy
Leipzig University
Johannisallee 29, 04103 Leipzig (Germany)
E-mail: hey@uni-leipzig.de
[b] *Dr. L. Kuhnert,[+] Prof. Dr. W. Honscha*
Institute of Pharmacology, Pharmacy and Toxicology
Faculty of Veterinary Medicine
Leipzig University
An den Tierkliniken 15, 04103 Leipzig (Germany)
[c] *S. George, Dr. B. Hofmann, Prof. Dr. D. Steinhilber*
Institute of Pharmaceutical Chemistry
University of Frankfurt
Max-von-Laue-Straße 9, 60438 Frankfurt (Germany)
[d] *Dr. D. Draca, Dr. S. Paskas, Prof. Dr. S. Mijatović, Prof. Dr. D. Maksimović-Ivanić*
Department of Immunology
Institute for Biological Research "Sinisa Stankovic"
University of Belgrade
Bul. despota Stefana 142, 11060 Belgrade (Serbia)
[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmdc.202100588

Results and Discussion

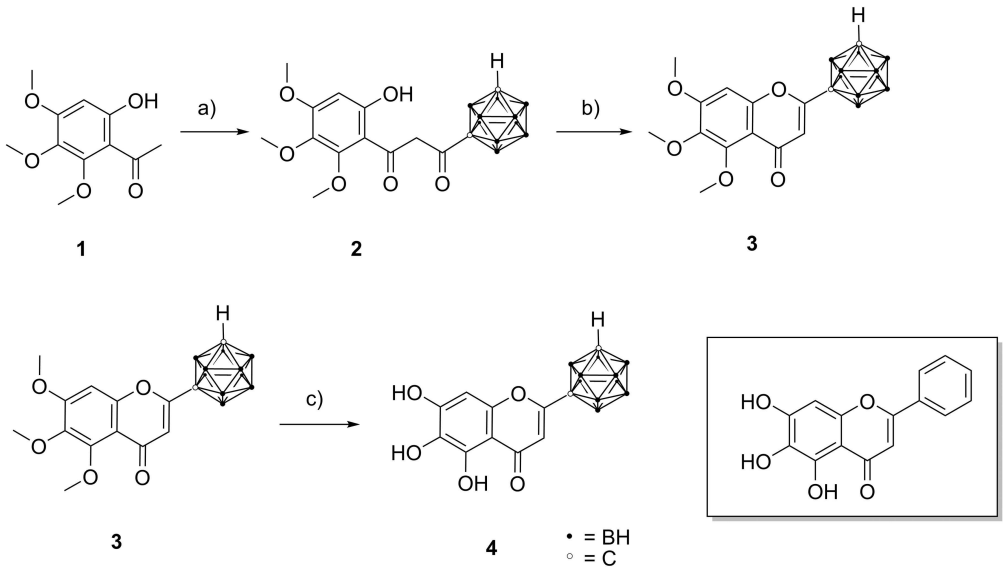
Syntheses

We have developed a suitable route for the introduction of *meta*-carborane into baicalein (Scheme 1). 1-(6-Hydroxy-2,3,4-trimethoxyphenyl)ethanone (**1**) has been prepared from commercially available 3,4,5-trimethoxyphenol as reported by Yoon et al.[16] The appropriate enolate, generated with two equivalents of freshly prepared lithium diisopropylamide, reacted with 1,7-dicarba-*closo*-dodecaboran(12)yl-1-carboxylic acid ethyl ester[17] yielding intermediate **2** which was not isolated. Compound **2** was refluxed with sulfuric acid in acetonitrile to obtain compound **3** (trimethoxyborcalein) via acid-catalysed cyclisation. The methoxy groups were deprotected with boron tribromide yielding compound **4** (borcalein), the *meta*-carborane analogue of baicalein.

Compounds **3** and **4** were fully characterised and their structures were elucidated by HMBC-NMR spectroscopy.

Evaluation of the inhibitory potential toward 12-LO

Borcalein (compound **4**) and its parental compound baicalein were investigated for their ability to inhibit 12-LO in comparison to the positive control nordihydroguaiaretic acid (NDGA). Homogenates of freshly isolated human platelets were used to detect an inhibition of 12-lipoxygenase. The inhibitory activity is represented by a decreased amount of the 12-LO metabolite 12(S)-H(p)ETE (as sum of 12(S)-HETE and 12-hydroperoxyeicosatetraenoic acid (12(S)-HpETE)). As presented in Figure 1, the positive control NDGA, baicalein and borcalein showed a reduction of 12-LO activity detected via decreased formation of 12(S)-H(p)ETE in human platelet homogenates.



Scheme 1. Synthesis of borcalein (compound **4**), the *meta*-carborane analogue of baicalein (framed). Reagents and conditions: (a) (1) lithium diisopropylamide, −50 °C, tetrahydrofuran; (2) 1,7-dicarba-*closo*-dodecaboran(12)yl-1-carboxylic acid ethyl ester, −50 °C to RT, tetrahydrofuran, 24 h. (b) H_2SO_4, MeCN, 70 °C, 24 h. (c) BBr_3, dichloromethane, −70 °C to RT, 24 h.

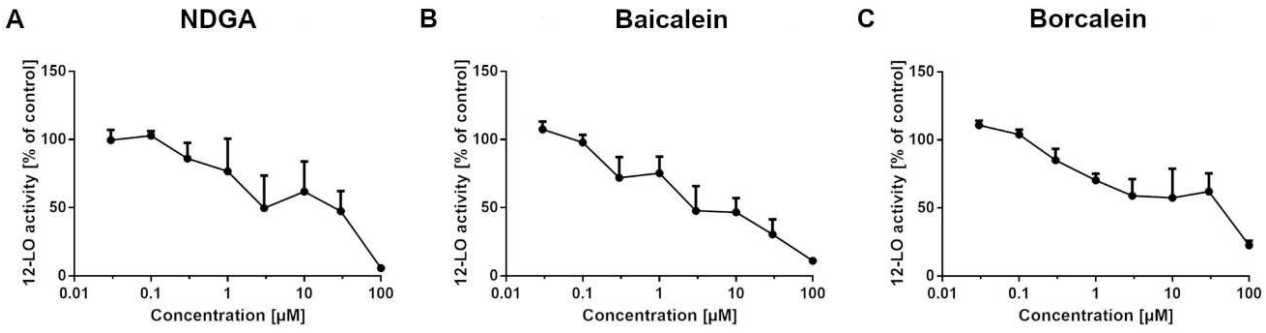


Figure 1. Inhibition of 12-lipoxygenase in human platelet homogenate. Cell homogenate was incubated with increasing concentrations of (A) the positive control NDGA, (B) baicalein and (C) borcalein (compound **4**). Formation of the 12-LO metabolite 12(S)-H(p)ETE was quantified by HPLC. An inhibition of 12-LO activity is represented by a decreased amount of 12(S)-H(p)ETE in comparison to the control treated with solvent (DMSO) only. Data are presented as mean ± SEM from three independent experiments.

The IC_{50} value obtained for baicalein was estimated as about 3–10 μM in homogenate (Figure 1). The inhibitory potential of baicalein is comparable to the positive control NDGA. Literature data for baicalein differ between 0.63 ± 0.11 μM[3] up to 64% inhibition at 100 μM[18] depending on experimental approach and analytical quantification.[6] For borcalein, an inhibition of the enzyme activity by 50% was determined at slightly above 30 μM. Overall, the IC_{50} value of baicalein and borcalein is estimated about 10 μM and 30 μM toward 12-LO in human platelet homogenate, respectively.

Molecular docking studies

In contrast to our expectation, our data showed a reduced inhibitory potential of the carborane-containing compound borcalein in comparison to baicalein. To explain the different inhibitory activity, both rigid and flexible *in silico* docking simulations were carried out with baicalein and borcalein as 12-LO ligands. In both docking simulations, the binding energy of baicalein toward 12-LO was lower than borcalein, reflecting a lower affinity of borcalein to the binding pocket of 12-LO (Table 1). Based on our molecular docking simulations, the bulky *meta*-carborane cluster prevents the formation of crucial interactions with 12-LO residues and the iron centre of 12-LO (see Supporting Information for details). Thus, the carborane cluster decreases the binding affinity and the inhibitory potential toward 12-LO in accordance with the *in vitro* experiments.

Table 1. Binding energies [$kJ \cdot mol^{-1}$] of baicalein and borcalein toward 12-LO in rigid and flexible docking simulations.

	baicalein	borcalein
rigid docking	−1.64	−1.13
flexible docking	−5.47	−4.07

Evaluation of the cytotoxicity against cancer cell lines

Since baicalein is a potent anti-tumour agent in higher doses, the cytotoxic behaviour of borcalein was investigated to determine potential beneficial anti-tumour effects in comparison to baicalein. The cytotoxic activity of borcalein on melanoma and colon cancer cell lines was determined to investigate whether borcalein affects cancer cell lines expressing lipoxygenase. Melanoma cell lines (A375, B16, B16F10) and colon carcinoma cells (HCT116, CT26CL25) were chosen,[19] and non-malignant MRC-5 cells, representing human lung fibroblasts, were used to calculate the selectivity index of baicalein and borcalein. Human colon carcinoma cell line SW480 with a low level of endogenous LO expression[20] was used to investigate LO-independent effects. After treating the cells with baicalein and borcalein in concentrations of 0.8 μM up to 50 μM for 48 h, the cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet (CV) assay (see Supporting Information for details).

As shown in Figure 2, IC_{50} values, representing a reduced cell viability of about 50%, were concordantly obtained from both assays. All tested compounds reduced the viability of melanoma and colon cancer cells (Supporting Information Figures S3 and S4) in a dose-dependent manner. In comparison to baicalein, borcalein significantly reduced the cell viability of non-malignant MRC-5 cells (Figure 2B) and all selected cancer cell lines (Supporting Information Figure S3 and S4). It is important to note that MRC-5 cells are a common model of non-malignant human cells with one crucial limitation: This cell line was isolated from human foetus 14 weeks old and belongs to highly proliferative embryonal cells.[21] Thus, a borcalein-treatment of adult mature cells may cause a lower cytotoxicity than detected in the higher proliferative MRC-5 cells. For baicalein and borcalein, we expected a higher selectivity against 12-LO positive cell lines, such as A375, B16 and B16F10.[22] In our experiments, baicalein and borcalein displayed the highest selectivity against the mouse colon carcinoma cell line

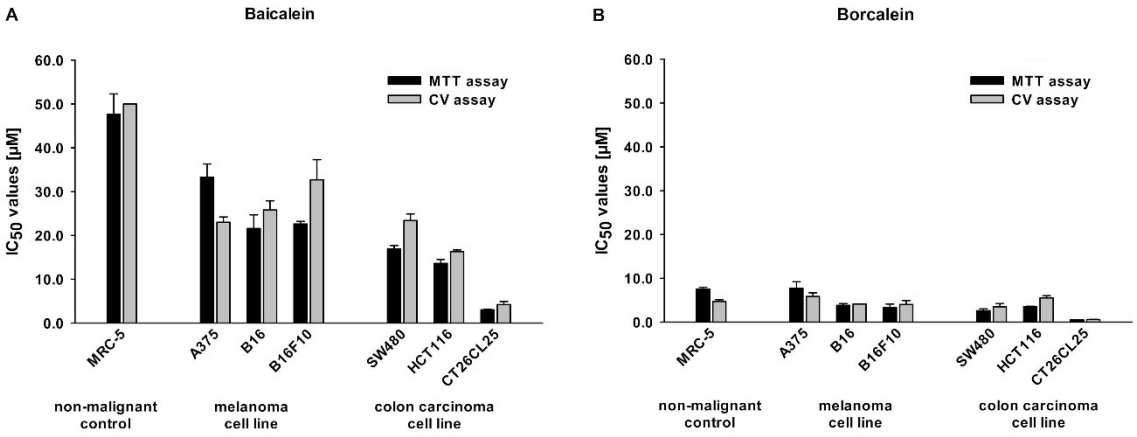


Figure 2. IC_{50} values of baicalein (**A**) and borcalein (**B**) on non-malignant MRC-5 lung fibroblasts, three different melanoma and colon carcinoma cell lines. The cytotoxicity of baicalein and borcalein was determined by MTT and CV assays in seven different cell lines (mean ± SEM, three independent experiments).

CT26CL25 with selectivity indices (IC_{50} MRC-5/IC_{50} tested compound) of 12.0 and 8.4, respectively, (Table 2) verifying the known anti-proliferative effect of baicalein on colorectal cancer cells.[23] Although a selectivity index of 3.0 is considered as highly selective,[24] borcalein displayed decreased tumour selectivity in all cell lines investigated in comparison to baicalein (Table 2). For all other cell lines investigated, borcalein has a comparable cytotoxicity to MRC-5 without tumour selectivity.

Since our aim was to improve the anti-tumour effects of baicalein by enhancing the drug stability and hydrophobicity via incorporation of a carborane moiety, the mode of cytotoxic activity of borcalein in comparison to baicalein was further investigated. The most sensitive cell line CT26CL25 was selected and cells were treated with IC_{50} concentrations of borcalein or baicalein for 48 h. Afterward, the impact of the compounds on proliferation rate, apoptosis, caspase activity as well as autophagy was assessed as described previously.[13,15,25]

In the carboxy fluorescein succinimidyl ester (CFSE) assay, a decreased fluorescence represents a higher proliferation rate of cells. A left shift of the signal on CFSE staining (decreased fluorescence) detected in the non-treated control indicates that CT26CL25 cells were dividing within 48 h (Figure 3A). In comparison, the fluorescence of baicalein- and borcalein-treated cells was higher (Figure 3A), representing a decreased dividing capacity of the CT26CL25 cells.

The annexin/propidium iodide staining (Ann/PI) was used to detect apoptotic CT26CL25 cells. Borcalein possessed a significantly higher potential to trigger apoptotic cell death measured by accumulation of early (Ann+/PI−) and late (Ann+/PI+) apoptotic cells in comparison to baicalein (Figure 3B). Although these compounds cause apoptosis, no caspase activation was observed in both cases by using apostat staining (Figure 3C), qualifying this apoptosis as caspase-independent. In concordance, a borcalein-mediated strong induction of autophagy was detected by acridine orange staining (Figure 3D). Observed autophagy was decreased in baicalein-treated cells (Figure 3D).

Either potentiated autophagy might represent the cellular attempt to restore the drug-induced damage, or it can contribute to overall drug toxicity as a type of programmed cell death.[26] Altogether, these data underline that borcalein has a stronger cytotoxic effect than baicalein manifested through increased inhibition of cell division, induction of caspase-independent apoptosis and autophagy.

Another known anti-tumour effect of baicalein is mediated by an induced production of reactive oxygen species[22,27] and this effect might also be involved in the enhanced cytotoxic activity of borcalein toward colon cancer cells. Hence, the

Table 2. Selectivity index values of borcalein and baicalein. The selectivity index was calculated as IC_{50} MRC-5/IC_{50} tested compound.

	Melanoma cell lines			Colon cancer cell lines		
Compd	A375	B16	B16F10	SW480	HCT116	CT26CL25
borcalein	0.8	1.2	1.2	1.3	0.8	8.4
baicalein	2.2	1.9	1.5	2.1	3.1	12.0

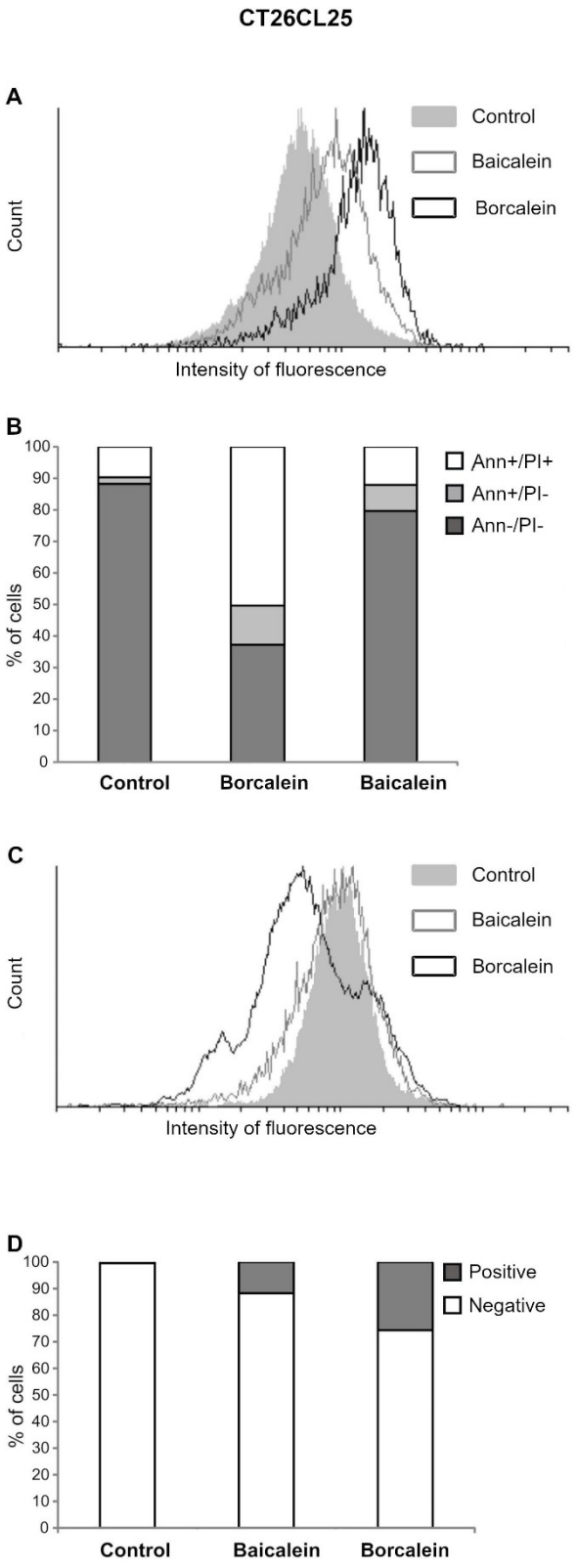


Figure 3. Mode of cytotoxicity of borcalein in comparison to baicalein. CT26CL25 cells were treated with IC_{50} doses of borcalein and baicalein for 48 h and then subjected to: (**A**) CFSE assay, (**B**) annexin/propidium iodide, (**C**) apostat, and (**D**) acridine orange staining. Cells were analysed by flow cytometry, one representative experiment out of three is shown.

production of reactive oxygen species (ROS)/reactive nitrogen species (RNS) as well as nitric oxide (NO) was additionally estimated as potential mediators of cytotoxicity.[13,15,28] ROS/RNS and NO production was measured by dihydrorhodamine (DHR) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) staining, respectively. Our results underline the production of ROS, RNS and NO by baicalein in CT26CL25 cells. Thus, the cytotoxic effects of baicalein might additionally be induced by ROS and not only via the inhibition of 12-LO. Indeed, the treatment of CT26CL25 cells with borcalein induced a significant release of reactive oxygen and nitrogen species, exceeding the amounts produced by baicalein-treated cells (Figure 4A). The obtained data suggest a direct involvement of oxidative burst in the borcalein-induced cytotoxicity.

Furthermore, for borcalein and baicalein we detected a significant intracellular amount of NO (Figure 4B) although these chemical structures do not contain nitric-oxide donors. Thus, the detected intracellular level of NO might be caused by an activation of endogenous NO production. This observation could be explained by activation of nitric-oxide synthase, as described by Tan et al.[29] in baicalein-treated cardiomyocytes. Borcalein was investigated for production of ROS, RNS and NO as well. It was shown that borcalein increased the production of ROS, RNS and NO much more than the parental compound baicalein. Thus, the observed cytotoxic effects of borcalein might be mediated by oxidative stress rather than via 12-LO inhibition as initially expected. Some 12-LO inhibitors are also known to disturb the redox homeostasis.[30] Flavones as baicalein have been shown to stabilise peroxide radicals to form intracellular hydrogen peroxide leading to apoptotic cell death.[31] Possibly, the electron-deficient carborane cluster enables an enhanced stabilisation and thus, an increased production of hydrogen peroxide in contrast to baicalein, leading to increased amounts of ROS resulting in an unselective toxicity by disturbance of redox homeostasis. Due to the increased toxicity toward non-malignant MRC-5, the introduction of carboranes into baicalein leads to decreased tumour selectivity.

Conclusion

12-Lipoxygenase (12-LO) is overexpressed in invasive cancer cells, such as prostate cancer or renal carcinoma, and stimulates angiogenesis by a 12(S)-HETE-mediated increase of the vascular endothelial growth factor (VEGF). Here we show the synthesis of borcalein (compound **4**), a carborane-based analogue of baicalein, as well as its inhibitory and cytotoxic potential. The replacement of the phenyl moiety by *meta*-carborane leads to increased cytotoxicity without an enhanced inhibition of 12-LO. Docking studies revealed the three-dimensional boron cluster to be sterically too demanding to increase the affinity to the binding pocket of 12-LO. Beside the 12-LO inhibition, the anti-tumour effect of baicalein is mediated by an increase of reactive oxygen and nitrogen species. In contrast to its parental compound, borcalein displayed an increased cytotoxicity with a decreased anti-cancer selectivity. Possibly, the remarkably higher production of reactive oxygen and nitrogen species in colon cancer cells exposed to borcalein may cause the non-specific toxic behaviour of the carborane-based analogue. Thus, an optimisation of carborane-based baicalein derivatives might be investigated for a selective activation of ROS/RNS-mediated anti-cancer effects.

Experimental Section

Syntheses

Materials and methods: All solvents were degassed, dried and purified with the solvent purification system SPS-800 by MBraun. All reactions were carried out under nitrogen using standard Schlenk techniques. The NMR spectra were recorded with a Bruker Avance DRX 400 spectrometer (^{1}H-NMR 400.13 MHz, ^{11}B-NMR 128.38 MHz, ^{13}C-NMR 100.63 MHz) at 25 °C. TMS was used as internal standard in the ^{1}H-NMR spectra and all other nuclei spectra were referenced to TMS using the Ξ-scale.[32] ^{13}C{^{1}H}-NMR spectra were recorded as APT spectra. Assignment of the chemical shifts was done using COSY, HMQC and HMBC techniques. ESI mass spectra were measured using a Bruker Daltonics APEX II FT-ICR spectrometer. IR spectra were obtained using an FTIR spectrometer (Genesis ATI, Mattson/Unicam) in the range of 400–4000 cm^{-1} in KBr. The melting points were determined in glass capillaries using a

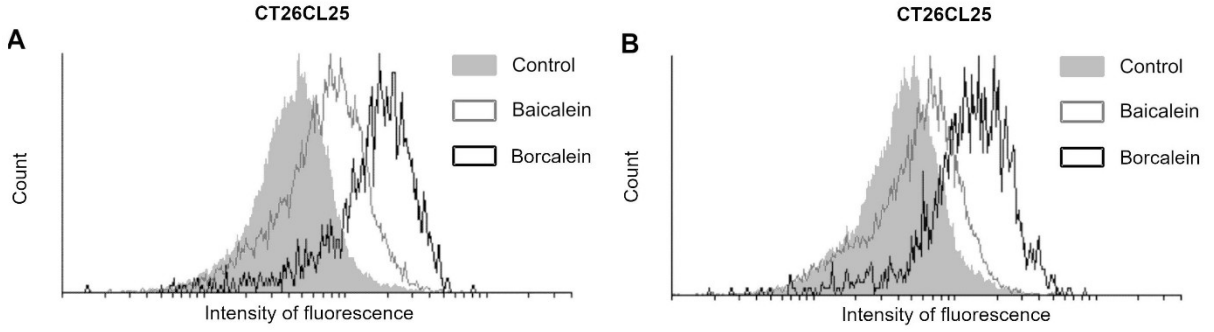


Figure 4. Baicalein and borcalein increase the production of ROS/RNS and NO. After treatment with IC_{50} doses of borcalein and baicalein for 48 h, cells were subjected to (**A**) dihydrorhodamine (DHR) or (**B**) 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) staining and analysed by flow cytometry to determine ROS/RNS and NO production, respectively. One representative experiment out of three is shown.

Gallenkamp apparatus and are uncorrected. Column chromatography was performed using a Biotage Isolera Four with ELSD-1080 detector and KP-SIL columns. 1,7-Dicarba-*closo*-dodecaborane(12) (Katchem, Prague, Czech Republic), baicalein (ABCR) and 5,6,7-trimethoxychromene-4-one (ABCR) are commercially available. 5,6,7-Trimethoxychromone has been prepared according to the literature.[16] All chemicals have been used without further purification.

2-(1,7-Dicarba-*closo*-dodecaboran(12)-1-yl)-5,6,7-trimethoxy-4*H*-chromen-4-one (compound 3): Diisopropylamine (2.10 eq., 2.74 mL, 1.96 g, 19.40 mmol) was dissolved in 100 mL tetrahydrofuran. The solution was cooled to 0 °C and *n*BuLi (2.15 eq., 1.45 M in *n*-hexane, 13.7 mL, 19.87 mmol) was added dropwise. After 30 min, the yellow solution was cooled to −50 °C and 1-(6-hydroxy-2,3,4-trimethoxyphenyl)ethanone[16] (1.00 eq., 2.09 g, 9.24 mmol) dissolved in 20 mL tetrahydrofuran was added dropwise. After 2 h at −50 °C, 1,7-dicarba-*closo*-dodecaboran(12)yl-1-carboxylic acid ethyl ester[17] (1.00 eq., 2.00 g, 9.24 mmol) dissolved in 20 mL tetrahydrofuran was added dropwise and the solution was warmed to room temperature and stirred overnight. Afterward the solution was warmed to 50 °C over 4 h. The solution was cooled to room temperature, 50 mL distilled water were added and the solution was acidified with concentrated hydrochloric acid. The aqueous phase was extracted 5 times with 50 mL diethyl ether and the combined organic phases were dried over magnesium sulfate. The solvent was removed and the residue was dissolved in 100 mL acetonitrile. Concentrated sulfuric acid (2.0 mL) was added and the solution was warmed to 70 °C and stirred overnight. The solution was cooled to room temperature, 50 mL distilled water were added and the aqueous phase was extracted three times with 50 mL dichloromethane. The combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (*n*-hexane/EtOAc, 3:1) and recrystallised from ethyl acetate. White solid, yield 11% (363 mg, 0.96 mmol); mp: 170–171 °C; TLC (*n*-hexane/EtOAc, 3:1): RF = 0.36; ^{1}H-NMR ($CDCl_3$): δ 6.65 (s, 1H), 6.28 (s, 1H), 3.95 (s, 3H), 3.92 (s, 3H), 3.87 (s, 3H), 3.10 (br s, 1H), 1.10–3.42 (br m, 10H); ^{11}B-NMR ($CDCl_3$): δ −4.4 (d, 1B, J_{BH} = 167.9 Hz), −6.9 (s, 1B, J_{BH} = 154.2 Hz), −10.5 (dd, 4B, J_{BH} = 159.7 Hz, 60.0 Hz), −13.2 (d, 2B, J_{BH} = 159.7 Hz), −15.5 (d, 2B, J_{BH} = 184.7 Hz); ^{13}C{^{1}H}-NMR ($CDCl_3$): δ 175.9, 158.2, 156.2, 154.2, 152.4, 140.8, 112.3, 111.5, 96.0, 71.6, 62.1, 61.4, 56.3, 54.9; IR (KBr, cm^{-1}): 3082, 3035, 2633, 1640, 1601, 1469, 1444, 1417, 1345, 1198, 1117, 1098, 828, 729; HR-ESI-MS (*m/z*): $[M+H]^+$ calculated for $C_{14}H_{23}B_{10}O_5$, 379.2559, found, 379.2551; Elemental analysis (% calculated, % found for $C_{14}H_{22}B_{10}O_5$): C (44.43, 44.62), H (5.86, 5.67).

2-(1,7-Dicarba-*closo*-dodecaboran(12)-1-yl)-5,6,7-trihydroxy-4*H*-chromen-4-one (compound 4): Boron tribromide (6.7 mL, 1 M in *n*-heptane, 6.70 mmol, 10.0 eq.) was added carefully to a solution of 255 mg (0.67 mmol, 1.0 eq.) compound **3** in 80 mL dichloromethane at −80 °C, and the solution was warmed to room temperature overnight. The resulting yellow suspension was cooled to 0 °C and 50 mL distilled water were added. The aqueous phase was extracted 5 times with 20 mL ethyl acetate. The combined organic phases were dried over magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (*n*-hexane/EtOAc, 1:2). Pale yellow solid, yield 40% (90 mg, 0.27 mmol); mp: 247–248 °C; TLC (*n*-hexane/EtOAc, 1:2): RF = 0.63; ^{1}H-NMR (acetone-d_6): δ = 12.22 (s, 1H), 9.22 (s, 1H), 8.15 (s, 1H), 6.57 (s, 1H), 6.27 (s, 1H), 3.98 (s, 1H), 0.71–3.14 (br m, 10H); ^{11}B-NMR (acetone-d_6): δ = −4.8 (d, 2B, J_{BH} = 168.8 Hz), −6.9 (s, 1B, J_{BH} = 165.2 Hz), −10.8 (d, 3B, J_{BH} = 156.7 Hz), −13.1 (d, 2B, J_{BH} = 169.5 Hz), −15.2 (d, 2B, J_{BH} = 187.4 Hz); ^{13}C{^{1}H}-NMR (acetone-d_6): δ = 181.4, 158.4, 153.4, 150.4, 146.7, 129.3, 108.0, 104.4, 93.8, 72.2, 55.8; IR (KBr, cm^{-1}): 3100, 3052, 2603, 1655, 1618, 1576, 1540, 1472, 1333, 1276, 1161, 1038, 994; HR-ESI-MS (*m/z*): $[M+H]^+$ calculated for $C_{11}H_{17}B_{10}O_5$, 337.2080, found, 337.2076; Elemental analysis (% calculated, % found for $C_{11}H_{16}B_{10}O_5$): C (39.28, 40.46), H (4.79, 5.01).

Biological data

Materials and methods: HPLC solvents were obtained from Merck (Darmstadt, Germany). Arachidonic acid (Peroxide free) was purchased from Cayman Chemical (MI, USA), CV (crystal violet) from Mol (Belgrade, Serbia), Annexin V-FITC from BD Pharmingen (San Diego, CA, USA), Apostat from RD (RD Systems, Minneapolis, MN USA), DHR (Dihydrorhodamine 123) from Thermo Fisher Scientific (Waltham, MA, USA), DAF-FM (4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate) from Molecular probe (Eugen, OR, USA) and acridine orange from Lab Modena (Paris, France). All other used reagents were purchased from Sigma (St. Louis, MO) and Sigma-Aldrich (Munich, Germany).

Platelet preparation and 12-LO product determination in platelet homogenate: Human platelets were freshly isolated from platelet concentrates obtained at the Deutsche Blutspendedienst (Frankfurt, Germany). Platelets were re-suspended in PBS, pH 5.4 and centrifuged at 1,849 *g* for 15 min at room temperature (RT). The cells were re-suspended in PBS/NaCl (PBS, pH 5.4 and 0.9% NaCl, 1:1 dilution) and centrifuged at 1,849 *g* for 10 min at RT. For determination of 12-LO product formation in cell homogenates, platelets (1×10^8) were re-suspended in 1 mL PBS (containing 1 mM EDTA) and cooled on ice for 5 min. After sonication (3×10 s), the test compounds were added (5 up to 10 min at 4 °C), the samples were preincubated for 30 s at 37 °C and the 12-LO product formation was stimulated by the addition of 2 mM calcium and 20 μM arachidonic acid. After 10 min at 37 °C, the reaction was stopped with 1 mL of ice-cold methanol. 12-LO products include 12(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12(S)-H(p)ETE) which eluted as one major peak and was analysed by HPLC a described previously.[33] Data (mean ± SEM) were expressed as percentage of control (DMSO). For 12-LO, the known lipoxygenase inhibitor NDGA (nordihydroguaiaretic acid) was used as control.

Cultivation of the cells: Human melanoma A375, mouse melanoma B16 and B16F10, human colon cancer HCT116 and SW480, mouse colon cancer CT26CL25 and non-malignant MRC-5 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS with 2 mM L-glutamine, 0.01% sodium pyruvate, 100 U/mL penicillin 100 μg/mL streptomycin (culture medium) at 37 °C in humidified atmosphere with 5% CO_2. After detaching by trypsinisation, cells were seeded in a density of 3×10^3 cells/well in 96-well plates and 2×10^4 cells/well in 24-well plates for viability assessment and flow cytometry, respectively. Baicalein and compound **4** (borcalein) were dissolved in DMSO and stored at −20 °C for a month.

Determination of cell viability by crystal violet (CV) and MTT assays: Cells were incubated with 0.8 up to 50 μM of selected compounds diluted in culture medium for 48 h and subsequently, cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and CV assays as described previously.[15,28] Solvent-treated cells served as control. Overall, cell viability of treated cells was normalised to control levels set as 100% and IC_{50} values were calculated. IC_{50} values are defined as substance-specific concentration decreasing cell viability to 50%.

Detection of cell proliferation by CFSE staining: Cells were stained with 1 μM of CFSE (carboxyfluorescein diacetate succinimidyl ester) for 10 min at 37 °C (5% pCO_2). Afterward, cells were treated with IC_{50} concentrations of the selected compounds for 48 h, and

analysed by flow cytometry (CyFlow® Space Partec and PartecFloMax® software) as described previously.[13,15]

Detection of the cell death by annexin V/propidium iodide staining and caspase activity by apostat staining: Cells were treated with IC_{50} doses of baicalein and borcalein for 48 h. Subsequently, to evaluate whether cytotoxic effects are mediated by induction of apoptosis or caspases, annexin V/propidium iodide double-staining, or apostat staining were performed following manufacturer's protocol. Flow cytometry (CyFlow® Space Partec and PartecFloMax® software) was used for analysis as reported previously.[13,15]

Detection of autophagy by acridine orange staining: After 48 h incubation of the cells, acridine orange was used to detect autophagy. Therefore, cells were incubated with 1 μg/mL acridine orange for 15 min at 37 °C (5% pCO_2), followed by washing, re-suspending in PBS, and flow cytometry analysis as mentioned above.

Determination of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by DHR staining: Cells were incubated with 1 μM of redox-sensitive dye DHR (dihydrorhodamine 123) for 20 min, and subsequently, treated with IC_{50} doses of baicalein and borcalein for 48 h. After washing the cells with PBS, generation of ROS and RNS were detected and analysed by flow cytometry (CyFlow® Space Partec and PartecFloMax® software).[15,25]

Measurement of intracellular nitric oxide (NO) by DAF-FM staining: After 48 hours treatment, cells were stained with 2 μM DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) in phenol red free RPMI-1640 medium for 1 h at 37 °C. Then, cells were treated and analysed as indicated previously.[15,28]

Statistics: Each applied substance concentration was normalised to vehicle control (DMSO) set as 100%. IC_{50} values (and confidence intervals) were calculated from at least three independent experiments using a nonlinear fit (log [inhibitor] vs normalised response – variable slope) by GraphPad Prism® version 6 (GraphPad Software, La Jolla, USA) software.

Docking studies

Computational details: Computations were carried out following a protocol published earlier.[13,34] Only crucial deviations from that protocol are listed here. The crystal structure of the catalytic domain of porcine leukocyte 12-lipoxygenase (PDB ID: 3RDE) was obtained from the Protein Data Bank (www.rcsb.org).[35] Ligands, all non-standard residues and all water molecules, including the water/hydroxide coordinated to the iron centre, were removed from the crystal structure. A $48\times44\times48$ Å^3 three-dimensional affinity grid was placed near the 12-LO catalytic centre. During the flexible docking simulations, the following 12-LO sidechains were allowed to move: F175, L179, E357, I400, L408, I414, F415, Q548, I593, Q596, L597, I663.

Acknowledgements

Financial support by the European Social Fund (ESF), the Graduate School Leipzig School of Natural Sciences – Building with Molecules and Nano-objects (BuildMoNa), Fonds der Chemischen Industrie, the Deutsche Forschungsgemeinschaft [DFG; SFB 1039, HE 1376/38-1, SA 2902/2-1 (to E.H.-H., R.K. and M.B.S.)], the Ministry of Education, Science and Technological Development of the Republic of Serbia (451-03-9/2021-14/200007) (S.M.,D.M.-I.) and the Else Kröner-Fresenius Foundation (D.S.) is gratefully acknowledged. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: carboranes · cancer · lipoxygenase · inflammation · inhibitors

[1] M. Sonoda, T. Nishiyama, Y. Matsukawa, M. Moriyasu, *J. Ethnopharmacol.* **2004**, *91*, 65–68.
[2] a) D. Steinhilber, *Lipoxygenases in Inflammation*, Springer International Publishing, **2016**; b) W.-Y. Gong, Z.-X. Zhao, B.-J. Liu, L.-W. Lu, J.-C. Dong, *Eur. J. Med. Chem.* **2017**, *126*, 844–852.
[3] J. D. Deschamps, V. A. Kenyon, T. R. Holman, *Bioorg. Med. Chem.* **2006**, *14*, 4295–4301.
[4] I. Winer, D. P. Normolle, I. Shureiqi, V. K. Sondak, T. Johnson, L. Su, D. E. Brenner, *Melanoma Res.* **2002**, *12*, 429–434.
[5] a) B. Liu, L. J. Marnett, A. Chaudhary, C. Ji, I. A. Blair, C. R. Johnson, C. A. Diglio, K. V. Honn, *Laboratoires* **1994**, *70*, 314–323; b) D. Nie, K. Tang, K. Szekeres, L. Li, K. V. Honn, *Ann. N. Y. Acad. Sci.* **2000**, *905*, 165–176.
[6] I. Schneider, F. Bucar, *Phytother. Res.* **2005**, *19*, 263–272.
[7] a) N. A. Alsharairi, *Int. J. Environ. Res. Public Health* **2021**, *18*, 5243–5260; b) L. Yang, Z. Wang, *Biomedicine* **2021**, *9*, 689–715; c) L. Wang, Y. Ling, Y. Chen, C.-L. Li, F. Feng, Q.-D. You, N. Lu, Q.-L. Guo, *Cancer Lett.* **2010**, *297*, 42–48; d) Y.-W. Chiu, T.-H. Lin, W.-S. Huang, C.-Y. Teng, Y.-S. Liou, W.-H. Kuo, W.-L. Lin, H.-I. Huang, J.-N. Tung, C.-Y. Huang, J.-Y. Liu, W.-H. Wang, J.-M. Hwang, H.-C. Kuo, *Toxicol. Appl. Pharmacol.* **2011**, *255*, 316–326; e) A.-M. Wang, H.-H. Ku, Y.-C. Liang, Y.-C. Chen, Y.-M. Hwu, T.-S. Yeh, *J. Cell. Biochem.* **2009**, *106*, 682–692; f) W.-S. Huang, Y.-H. Kuo, C.-C. Chin, J.-Y. Wang, H.-R. Yu, J.-M. Sheen, S.-Y. Tung, C.-H. Shen, T.-C. Chen, M.-L. Sung, H.-F. Liang, H.-C. Kuo, *Proteomics* **2012**, *12*, 810–819; g) Z. Guo, X. Hu, Z. Xing, R. Xing, R. Lv, X. Cheng, J. Su, Z. Zhou, Z. Xu, S. Nilsson, Z. Liu, *Mol. Cell. Biochem.* **2015**, *406*, 111–119.
[8] F.-R. Wang, Y.-S. Jiang, *J. Neuro-Oncol.* **2015**, *124*, 5–11.
[9] a) R. Ancuceanu, M. Dinu, C. Dinu-Pirvu, V. Anuța, V. Negulescu, *Pharmaceutica* **2019**, *11*; b) L. Zhang, C. Li, G. Lin, P. Krajcsi, Z. Zuo, *AAPS J.* **2011**, *13*, 378–389; c) B. Zhang, Y. Dong, N. Yu, Y. Sun, Y. Xing, F. Yang, X. Yu, W. Sun, J. Sun, X. Li, Z. Xiu, *J. Funct. Foods* **2019**, *54*, 53–63.
[10] a) M. Scholz, A. L. Blobaum, L. J. Marnett, E. Hey-Hawkins, *Bioorg. Med. Chem.* **2012**, *20*, 4830–4837; b) R. N. Grimes, *Carboranes*, Third ed., Elsevier, Academic Press, Amsterdam, Boston, Heidelberg, **2016**; c) I. B. Sivaev, V. V. Bregadze, *Eur. J. Inorg. Chem.* **2009**, 1433–1450; d) F. Issa, M. Kassiou, L. M. Rendina, *Chem. Rev.* **2011**, *111*, 5701–5722.
[11] P. Stockmann, M. Gozzi, R. Kuhnert, M.-B. Sárosi, E. Hey-Hawkins, *Chem. Soc. Rev.* **2019**, *48*, 3497–3512.
[12] M. Scholz, E. Hey-Hawkins, *Chem. Rev.* **2011**, *111*, 7035–7062.
[13] R. Kuhnert, M.-B. Sárosi, S. George, P. Lönnecke, B. Hofmann, D. Steinhilber, B. Murganić, S. Mijatović, D. Maksimović-Ivanić, E. Hey-Hawkins, *ChemMedChem* **2017**, *12*, 1081–1086.
[14] W. Neumann, S. Xu, M.-B. Sárosi, M. S. Scholz, B. C. Crews, K. Ghebreselasie, S. Banerjee, L. J. Marnett, E. Hey-Hawkins, *ChemMedChem* **2016**, *11*, 175–178.
[15] R. Kuhnert, M.-B. Sárosi, S. George, P. Lönnecke, B. Hofmann, D. Steinhilber, S. Steinmann, R. Schneider-Stock, B. Murganić, S. Mijatović, D. Maksimović-Ivanić, E. Hey-Hawkins, *ChemMedChem* **2019**, *14*, 255–261.
[16] H.-J. Yoon, M.-K. Kim, H.-J. Mok, Y.-H. Chong, *Bull. Korean Chem. Soc.* **2012**, *33*, 2803–2805.
[17] K. B. Gona, J. L. V. N. P. Thota, Z. Baz, V. Gómez-Vallejo, J. Llop, *Molecules* **2015**, *20*, 7495–7508.
[18] K. M. You, H. G. Jong, H. P. Kim, *Arch. Pharmacal Res.* **1999**, *22*, 18–24.
[19] K.-H. Kang, T.-Y. Ling, H.-H. Liou, Y.-K. Huang, M.-J. Hour, H.-C. Liou, W.-M. Fu, *Eur. J. Cancer* **2013**, *49*, 2747–2759.
[20] T. Klampfl, E. Bogner, W. Bednar, L. Mager, D. Massudom, I. Kalny, C. Heinzle, W. Berger, S. Stättner, J. Karner, M. Klimpfinger, G. Fürstenberger, P. Krieg, B. Marian, *Exp. Cell Res.* **2012**, *318*, 768–778.

[21] J. P. Jacobs, C. M. Jones, J. P. Baille, *Nature* **1970**, *227*, 168–170.
[22] D.-S. Chou, G. Hsiao, Y.-A. Lai, Y.-J. Tsai, J.-R. Sheu, *Free Radical Biol. Med.* **2009**, *46*, 1197–1203.
[23] a) J. Yáñez, V. Vicente, M. Alcaraz, J. Castillo, O. Benavente-García, M. Canteras, J. A. L. Teruel, *Nutr. Cancer* **2004**, *49*, 191–199; b) S.-J. Kim, H.-J. Kim, H.-R. Kim, S.-H. Lee, S.-D. Cho, C.-S. Choi, J.-S. Nam, J.-Y. Jung, *Mol. Med.* **2012**, *6*, 1443–1449.
[24] M. Michalak, M. S. Lach, M. Antoszczak, A. Huczyński, W. M. Suchorska, *Molecules* **2020**, *25*, 537–552.
[25] D. Maksimović-Ivanić, S. Mijatović, L. Harhaji, D. Miljković, D. Dabideen, K. Fan Cheng, K. Mangano, G. Malaponte, Y. Al-Abed, M. Libra, G. Garotta, F. Nicoletti, S. Stošić-Grujičić, *Mol. Cancer Ther.* **2008**, *7*, 510–520.
[26] a) G. Mariño, M. Niso-Santano, E. H. Baehrecke, G. Kroemer, *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 81–94; b) P. Tsapras, I. P. Nezis, *Cell Death Differ.* **2017**, *24*, 1369–1379.
[27] M. Li-Weber, *Cancer Treat. Rev.* **2009**, *35*, 57–68.
[28] S. Mijatović, D. Maksimović-Ivanić, J. Radović, D. Popadić, M. Momčilović, L. Harhaji, D. Miljkovic, V. Trajkovic, *Cell. Mol. Life Sci.* **2004**, *61*, 1805–1815.
[29] S. Tan, S. Zhou, Y. Luo, *Mol. Med.* **2014**, *9*, 2429–2434.
[30] H. Kuhn, S. Banthiya, K. van Leyen, *Biochim. Biophys. Acta* **2015**, *1851*, 308–330.
[31] S. Zhang, Y. Bao, X. Ju, K. Li, H. Shang, L. Ha, Y. Qian, L. Zou, X. Sun, J. Li, Q. Wang, Q. Fan, *Sci. Rep.* **2015**, *5*, 13626.
[32] R. K. Harris, E. D. Becker, S. M. Cabral De Menezes, R. Goodfellow, P. Granger, *Concepts Magn. Reson.* **2002**, *14*, 326–346.
[33] M. Brungs, O. Radmark, B. Samuelsson, D. Steinhilber, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 107–111.
[34] M. D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeersch, E. Zurek, G. R. Hutchison, *J. Cheminf.* **2012**, *4*, 17.
[35] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235–242.

Manuscript received: September 8, 2021
Revised manuscript received: October 23, 2021
Accepted manuscript online: October 25, 2021
Version of record online: November 11, 2021