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ATG4B inhibitors with a benzotropolone core structure block autophagy and augment efficiency of chemotherapy in mice

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Running title: ATG4B inhibitors impair tumor growth

Abstract

Autophagy is a cell survival mechanism hijacked by advanced tumors to endure a rough microenvironment. Late autophagy inhibitors such as (hydroxy)chloroquine have been used clinically to halt tumor progression with modest success. However, given the toxic nature of these compounds and their lack of specificity, novel targets should be considered. We recently identified a benzotropolone derivative that significantly inhibited the essential autophagy protein ATG4B. Therefore, we synthesized and tested additional benzotropolone compounds to identify a promising ATG4B inhibitor that impairs autophagy both *in vitro* and *in vivo*. A compound library containing 27 molecules with a benzotropolone backbone was synthesized and screened for inhibition of recombinant ATG4B. Depending on the benzotropolone compound, inhibition of recombinant ATG4B ranged from 3 to 82%. Active compounds were evaluated in cellular assays to confirm inhibition of ATG4B and suppression of autophagy. Seven compounds inhibited processing of the autophagy protein LC3 and autophagosome formation. Compound UAMC-2526 was selected for further *in vivo* use because of its fair plasma stability. This compound abolished autophagy both in nutrient-deprived GFP-LC3 mice and in CD1^{-/-} Foxn1^{nu} mice bearing HT29 colorectal tumor xenografts. Moreover, addition of UAMC-2526 to the chemotherapy drug oxaliplatin significantly improved inhibition of tumor growth. Our data indicate that suppression of autophagy via ATG4B inhibition is a feasible strategy to augment existing chemotherapy efficacy and to halt tumor progression.

Keywords: ATG4B, autophagy, osmotic minipump, colorectal tumor, benzotropolone.

Compounds: Propylene glycol (PubChem CID: 1030), DMSO (PubChem CID: 679), Ethanol (PubChem CID: 702), Tween 20 (PubChem CID: 443314), Everolimus (PubChem CID: 6442177), 3-Methyladenine (PubChem CID: 1673), Chloroquine (PubChem CID: 2719), Bafilomycin A1 (PubChem CID: 6436223), Oxaliplatin (PubChem CID: 5310940), Sevoflurane (PubChem CID: 5206).

1. Introduction

Autophagy is a highly conserved intracellular process by which double membraneous vacuoles, known as autophagosomes, engulf cytoplasmic material including organelles and macromolecules for bulk degradation in lysosomes. It occurs constitutively under normal conditions and contributes to the intracellular housekeeping of the organism. (1–3) Under stress conditions (e.g. hypoxia, nutrient or growth factor deprivation, DNA damage), autophagy is highly stimulated to sustain energy production and to promote cell survival.(4,5) Along these lines, tumor cells use autophagy in response to hypoxia and chemotherapy to withstand the harsh microenvironment and to evade cell death.(6–10) Recent evidence suggests that autophagy has a dual role in oncogenesis and tumor progression. While autophagy is decreased in the early tumor stages, it is highly (re)activated in established tumors.(11,12) As such, the well-known autophagy marker microtubule-associated protein 1 light chain 3B (LC3B) is often increased in tumors and is predictive of poor prognosis.(13–17) Moreover, a role for autophagy in tumor metastasis has recently been suggested.(18) Multiple preclinical studies with very promising results have been conducted to evaluate autophagy inhibition as a novel target for cancer treatment.(19–21) These findings led to the initiation of several clinical trials featuring hydroxychloroquine, a less toxic derivative of the late autophagy inhibitor chloroquine, to enhance the effectiveness of chemotherapy.(22–27)

Chloroquine and its derivatives block autophagic flux by indifferent inhibition of lysosome proteases and are therefore unable to abrogate the formation of autophagosomes.(27,28) Other molecules such as 3-methyladenine (3MA) and wortmannin inhibit class III phosphatidylinositol-3-kinases (PI3Ks) to abolish autophagosome formation.(29) However, because PI3K is a regulator of many essential processes, its inhibition may lead to serious side effects rendering this approach unsuitable *in vivo*. Overall, we may conclude that the currently

available autophagy blockers are highly unspecific and lack potency and are hence limited in their use by severe toxicity.(28,30–32)

A critical component of the autophagy machinery is the cysteine protease ATG4 (autophagy related 4 cysteine protease). Among the four distinct forms identified in mammals, only ATG4B has been shown to have an important role in autophagy.(33) The enzyme converts pro-LC3B to LC3B-I by cleaving its C-terminus after which LC3B-I can be conjugated to phosphatidylethanolamine by other ATGs, thereby forming LC3B-II.(34) This lipidation process of LC3B-I allows expansion and closure of autophagosomes.(35) Given the encouraging results following ATG4B inhibition in several tumors,(36–38) the enzyme is becoming increasingly attractive as a therapeutic target for cancer,(39,40) and considerable efforts have recently been made to identify small molecule ATG4B inhibitors.(37,41–43)

Using recombinant ATG4B enzyme and a fusion protein of LC3 and glutathione-S-transferase (LC3-GST) substrate in a gel-based densitometric quantification assay, we recently found a novel compound with a benzotropolone core structure that significantly inhibited ATG4B activity.(44) To further optimize this lead compound, we synthesized 27 derivatives and used a variety of techniques to pinpoint the most promising autophagy inhibitor that was able to abolish ATG4B protease activity both *in vitro* and *in vivo*. Furthermore, the effect of autophagy inhibition on tumor growth and sensitivity to chemotherapy was investigated in a preclinical colorectal cancer xenograft model.

2. Materials and methods

2.1 Synthesis of benzotropolone structures

All benzotropolones evaluated in this manuscript were synthesized according to the following general procedures (Figure 1, Table 1):

General procedure A: oxidative synthesis of Target Compounds S-3 with $R^{4-6} = H$

Catechol derivative S-1 (1 molar eq.) and gallic acid derivative S-2 (1 molar eq.) were dissolved in a mixture of 5 mL acetone (Fisher Scientific, Hampton, USA) and 20 mL water (Milli-Q Direct 8, Millipore, MA, USA). A solution of 1 molar eq. potassium iodate (Sigma-Aldrich, MO, USA) in 20 mL water was added dropwise to the reaction mixture over the course of 1 hour while stirring at room temperature. The mixture was stirred for an extra 1.5 h after addition of the potassium iodate and was then left standing for 30 minutes, allowing the target compound to precipitate. The reaction mixture was filtered and the precipitate was washed 3 times with 1 M HCl (Sigma-Aldrich, MO, USA). After drying under high vacuum, the desired benzotropolone was obtained. If necessary, further purification steps were executed, consisting either of normal phase flash chromatography using acetonitrile (Sigma-Aldrich, MO, USA) as the eluent or recrystallization from acetonitrile/water. Catechol and gallate derivatives used in this step were either obtained from commercial sources or synthesized using standard literature procedures. Yields varied between 25% and 72 %.

General procedure B: preparation of methylated Target Compounds S-4 with $R^{4-6} = Me$

The required benzotropolone S-3 was suspended in 8 mL of anhydrous DMF (Sigma-Aldrich, MO, USA), as well as 12 eq. of sodium hydroxide (Sigma-Aldrich, MO, USA). The reaction mixture was maintained at 0°C under nitrogen, and 8 eq. of iodomethane (Sigma-Aldrich, MO, USA) were added. The mixture was stirred overnight at room temperature. Afterwards, the

reaction was quenched with 10 mL water and stirred for another 20 minutes. 20 mL of EtOAc (Fisher Scientific, Hampton, USA) was added and the obtained mixture was washed with 3 x 20 mL water. The aqueous phases were pooled and extracted with 20 mL EtOAc. Residual water was removed by drying over Na₂SO₄ (Sigma-Aldrich, MO, USA) and evaporating to further dryness under high vacuum. The crude was further purified by flash chromatography on silica gel, using EtOAc/Hexane (1:5) (Fisher Scientific, Hampton, USA) as eluent. The desired 2,3,4,6-tetramethoxy-5H-benzo[7]annulen-5-one was obtained as an amorphous powder (Yields: 78-89 %).

2.2 SDS-PAGE-based LC3-GST cleavage assay

LC3-GST and ATG4B were produced in *E. coli* and purified as previously described.(39,44) Next, an SDS-PAGE-based screening assay for ATG4B inhibition was performed.(44) Briefly, aliquots of purified LC3-GST and ATG4B were diluted in a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM DTT, 0.1% tween 20 to achieve a concentration of 0.625 ng/μL ATG4B and 0.4 μg/μL LC3-GST. Potential ATG4B inhibitors (dissolved in DMSO) were added to the test assay and samples were incubated at room temperature for 6 minutes. To stop the reaction, 10 μL of Laemmli sample buffer (Bio-Rad, CA, USA) was added. Samples were subsequently boiled for 4 minutes, loaded on NuPAGE 12% Bis-Tris gels (Invitrogen, CA, USA) and subjected to SDS-PAGE. Finally, gels were stained using OrioleTM Fluorescent Gel Stain (Bio-Rad, CA, USA) and scanned by a Lumi-Imager (Roche Diagnostics, Mannheim, Germany). Protein bands were quantified using Totallab Quant software. ATG4B inhibition was calculated using the following formula:

$$\text{enzyme inh. (\%)} = \frac{\% \text{ uncleaved}([LC3B-GST])_{\text{test sample}} - \% \text{ uncleaved}([LC3B-GST])_{\text{control sample}}}{100 \% - \% \text{ uncleaved}([LC3B-GST])_{\text{control sample}}} \times 100\%$$

2.3 Flow cytometry

Jurkat T-cells were cultured in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS). A total of 1×10^6 cells (passage # < 10) was transferred in the wells of a standard 12-well plate. Cells were treated with 10 μ M of the autophagy inducer everolimus in the presence or absence of 10 μ M test compound. 10 mM of the commonly used autophagy inhibitor 3MA served as a control. After 16 hours of treatment, cells were washed and autophagosomes were stained for 30 minutes with CYTO-ID Green Autophagy Detection Reagent (Enzo Life Sciences, NY, USA) according to the manufacturer's instructions. Finally, cells were washed and analyzed by flow cytometry using an Accuri c6 instrument (BD Biosciences, CA, USA).

2.4 Western blotting

Jurkat T-cells were treated as described in section 2.3 with the exception of bafilomycin A1 (Santa-Cruz, CA, USA) addition. Cells were then lysed in an appropriate volume of Laemmli buffer. Mouse tissue samples were first homogenized in RIPA buffer (Sigma-Aldrich, MO, USA) containing protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany) using a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Samples were then heat-denatured for 5 minutes in boiling water and loaded on pre-casted Novex Bolt 4-12% Bis-Tris gels (Invitrogen, CA, USA). After electrophoresis, proteins were transferred to an Immobilon-FL PVDF membrane (Millipore, MA, USA) according to standard procedures. Membranes were blocked for one hour in Odyssey blocking buffer (LI-COR Biosciences, ME, USA) diluted 1:5 with PBS. After blocking, membranes were probed overnight at 4 °C with primary antibodies diluted in Odyssey blocking buffer, followed by one hour incubation with IRDye-labeled secondary antibodies at room temperature. The following antibodies were used: mouse anti- β -actin (clone AC-15, Sigma-Aldrich, MO, USA,

A5441), mouse anti-LC3B (clone 5F10, Nanotools, Munich, Germany, 0231-100/LC3-5F10), rabbit anti-GFP (Abcam, Cambridge, UK, ab6556), rabbit anti-SQSTM1 (Cell Signaling Technology, MA, USA, #5114), rabbit anti-p70S6K (Cell Signaling Technology, MA, USA, #9202) and rabbit anti-phospho-p70S6K(Thr389) (Cell Signaling Technology, MA, USA, #9205). IRDye-labeled secondary antibodies (goat anti-mouse IgG, 926-68070 and goat anti-rabbit IgG, 926-32211) were purchased from LI-COR Biosciences, ME, USA. Antibody detection was achieved using an Odyssey SA infrared imaging system. The intensity of the protein bands was quantified using Image Studio software (LI-COR Biosciences, ME, USA).

2.5 *In vitro* ATG4B activity assay

HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Invitrogen, CA, USA) supplemented with 10% FBS. An Amaxa Cell Line Nucleofector kit V (Lonza, MD, USA, VCA-1003) was used to transfect 2×10^6 cells (passage # < 10) with either 3 μ g pEAK12 plasmid DNA encoding truncated *Gaussia* luciferase (dNGLUC) fused to β -actin and LC3 (β -actine-LC3-dNGLUC)(42) or 3 μ g control plasmid (pMaxGFP) according to standard procedures. After transfection, cells were transferred to a 24-well plate and incubated at 37 °C for 24 hours. Hereafter, culture medium was removed and 500 μ L serum-free RPMI medium containing 10 μ M everolimus with or without 10 μ M of test compound was added. Cells were incubated for 6 hours after which luciferase activity in the medium was assessed using a BioLux *Gaussia* Luciferase Assay kit (New England Biolabs, MA, USA). Briefly, medium (10 μ L) was transferred in an opaque 96-well microtiter plate and mixed with 50 μ L assay buffer. Luciferase activity was measured after a 5-second integration time using an Infinite® 200 Pro microplate reader (Tecan, Zurich, Switzerland).

2.6 Plasma stability assay

A 5 μ L aliquot of compound solution (10 mM in DMSO) was added to 995 μ L of Non Swiss Albino Mouse Plasma (DivBioScience, Breda, Netherlands) in sodium citrate to obtain a final concentration of 50 μ M inhibitor in plasma. The mixture was gently shaken for 6 hours at 37°C. Aliquots of 100 μ L were taken at various time points (0, 30, 60, 120, 180 and 360 minutes) and diluted with 400 μ L of cold methanol (Sigma-Aldrich, MO, USA) (stored at 4°C). The suspension was centrifuged at 400 x g for 5 minutes. Thereafter, 50 μ L of the supernatant was diluted with 950 μ L of methanol and analyzed with LC-MS/MS (Waters Acquity H-class UPLC system with ion trap mass spectrometer, Bruker Daltonics Esquire 3000 plus, and Agilent 1100 Series LC system). Samples were analyzed in triplicate and plotted against a standard curve (compound at 3.12-100 μ M in mouse plasma).

2.7 Microsomal stability assay

A mixture of 713 μ L milliQ water (Milli-Q Direct 8, Millipore, MA, USA), 200 μ L 0.5 M phosphate buffer (BD Diagnostic Systems, MD, USA), 50 μ L NADPH regenerating system solution A (BD Biosciences, CA, USA), 10 μ L NADPH regenerating system solution B (BD Biosciences, CA, USA) and 2 μ L compound (5 mM in DMSO) was prepared and heated for 5 minutes at 37°C. Twenty five μ L of liver microsomes (Corning B.V. Life Sciences, Amsterdam, Netherlands) was added to the mixture and 20 μ L samples were withdrawn at 0, 15, 30, 60, 120, 240 and 360 minutes. Eighty μ L (4 x 20 μ L) of cold acetonitrile (Sigma-Aldrich, MO, USA) was added to the samples on ice for 10 minutes. Afterwards, the mixtures were centrifuged at 400 x g for 5 minutes at 4°C. Seventy five μ L of an acetonitrile/water (10/90) mixture was added to 25 μ L supernatant and the obtained samples were analyzed by LC-MS/MS in triplicate.

2.8 Mice

GFP-LC3 transgenic mice, which contain green fluorescent protein (GFP) fused to rat microtubule-associated protein light chain 3 (LC3) and which are widely used for autophagy research(45) were obtained from the RIKEN BioResource Center (strain GFP-LC3 #53). CD1^{-/-} Foxn1^{nu} mice, which are used in the HT29 tumor xenograft experiments, were obtained from Charles River Laboratories. The animals had a weight of 26 ±3g and were kept under environmentally controlled conditions (12 h light/dark cycle, 20–24°C and 40–70% relative humidity) with food and water ad libitum. Mice were selected randomly from the pool of all cages and divided into groups. All analyses were carried out without prior knowledge of the treatments. Mice received a numerical code throughout the entire experiment. The study was approved by the ethics committee of the University of Antwerp (No. 2016-04).

2.9 *In vivo* autophagy inhibition in a healthy mouse model

Test compound was diluted in a vehicle composed of DMSO, propylene glycol and ethanol (50:40:10) supplemented with 0.4 µL/mL Tween 20 and loaded into osmotic mini pumps (model 1003D, Alzet, Charles River Laboratories, L'Arbresle, France) to achieve an *in vivo* pumping rate of 2 mg/kg/day. Male GFP-LC3 mice (n = 14, 3 months old) were divided into two groups, one group receiving osmotic mini pumps loaded with vehicle and a second group receiving mini pumps with test compound. Mice were anesthetized using Sevoflurane® (Abbott, IL, USA). After 24 hours, mice were starved overnight to induce autophagy. Following starvation, chloroquine was administered (100 mg/kg, I.P.) to inhibit autophagic flux and mice were sacrificed 3 hours later using an overdose of pentobarbital (250 mg/kg, I.P.). Liver samples were isolated for western blotting, immunohistochemistry and transmission electron microscopy.

2.10 *In vivo* autophagy inhibition in a tumor xenograft model

HT-29 (human colon adenocarcinoma, ATCC) cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin (Gibco, Life technologies, CA, USA). After detaching the cells, 10^7 viable cells (suspended in 100 μ l PBS) were inoculated in the right hind leg of CD1^{-/-} Foxn1nu mice. When tumors reached a volume of approximately 200 mm³, mice were divided into 4 groups (11 mice per group) for treatment with clinical-grade oxaliplatin (Eloxatin, Sanofi, Paris, France), test compound UAMC-2526, the combination of oxaliplatin and test compound, or vehicle. Oxaliplatin (5 mg/kg) was administered I.P. once a week for 28 days. Unidose solutions of compound UAMC-2526 were prepared in the previously described vehicle according to individual mouse weight and loaded into snap-fit-sealed polypropylene microvials. Solutions were prepared the day before treatment and transferred to osmotic minipumps (model 1002, Alzet, Charles River Laboratories, L'Arbresle, France) achieving an *in vivo* pumping rate of 3 mg/kg/day for 2 weeks. The osmotic minipumps were replaced after 2 weeks to obtain a total treatment period of 28 days. Mice that were not treated with oxaliplatin received an I.P. injection with saline at the same time. Body weight was measured twice a week and tumor growth was monitored three times a week with digital calliper measurements starting upon detection of palpable tumors. Tumor volume was calculated using the modified ellipsoid formula (i.e., $\frac{1}{2}(\text{length} \times \text{width}^2)$). The percentage of tumor growth inhibition (TGI) was calculated as $\left(1 - \frac{\text{RTV}_x}{\text{RTV}_c}\right) \times 100$, with RTV_x being the relative tumor volume from the treated group and RTV_c the relative tumor volume from the control group. The delay in tumor growth was calculated as the difference in time for control and treated tumors to grow from 200 mm³ to 800 mm³. Blood and tumor samples were isolated on day 28 or when tumors reached a volume of 1500 mm³.

2.11 *In vitro* proliferation assay

HT-29 cells were incubated overnight at a density of 6000 cells per well in 96-well plates after which UAMC-2526 (diluted in the vehicle described above) was added at different concentrations in the presence or absence of 10 μ M oxaliplatin. Cell proliferation was measured using WST-1 reagent (Roche Diagnostics, Mannheim, Germany) according to the instructions of the manufacturer.

2.12 Immunohistochemistry

Tissue samples were fixed in 4% neutral buffered formalin for 24 hours prior to paraffin embedding. Thereafter, 5 μ m thick sections were prepared and immunostained for LC3 using mouse anti-LC3 (clone 5F10, Nanotools, Munich, Germany, 0231-100/LC3-5F10) followed by anti-mouse Envision+ (Dako, Glostrup, Denmark) as previously described.(46,47) Quantification was done by taking 5 images per section at random and ImageJ software was used for analysis of the LC3-positive area. Tumor sections (5 μ m) were stained with hematoxylin-eosin to assess general tumor morphology and to quantify the percentage tumor necrosis. To assess cell proliferation and apoptosis, tumor sections were incubated overnight at 4°C with primary antibodies against Ki67 (Cell Signaling Technology, MA, USA #9027) and cleaved caspase-3 (Cell Signaling Technology, MA, USA #9664), respectively. Two independent observers including a certified pathologist performed the Ki67 staining, which was evaluated by assigning to each tumor a percentage of positively stained nuclei. Cleaved caspase-3 staining was scored using a customized script in ImageJ software. Positive staining was quantified as area fraction per ROI on whole tumor sections, not taking into account areas of high necrosis and edges of the tissue.

2.13 Fluorescence microscopy

Formalin-fixed, paraffin-embedded tissue sections (5 μm thick) were deparaffinized with toluene followed by washing in isopropanol and water. Samples were finished with Vectashield mounting medium containing DAPI (Vector laboratories, CA, USA, H-1200). Images were taken using an EVOS FL Auto Cell Imaging System (ThermoFisher Scientific, MA, USA) and quantification of GFP puncta was done with ImageJ software.

2.14 Transmission electron microscopy

Tissue samples were examined via transmission electron microscopy as previously described.⁽⁴⁶⁾ A FEI Tecnai microscope was used to examine ultrathin sections at 80-120 kV. Autophagic vacuoles were quantified on 5 different images taken at random per mouse.

2.15 Statistical analyses

Results are expressed as mean $\pm$ SEM. Statistical analyses were performed using SPSS software (version 24). The appropriate statistical test is reported in each figure legend. A probability value < 0.05 was considered significant.

3. Results

3.1 *In vitro* experiments

3.1.1 *In vitro* inhibition of ATG4B activity by benzotropolone derivatives

Twenty seven benzotropolone derivatives were evaluated for autophagy/ATG4B inhibition using a previously described gel-based LC3-GST cleavage assay.⁽⁴⁴⁾ Each test compound was incubated with LC3-GST substrate and ATG4B in Tris-HCl buffer for 6 minutes. Afterwards, residual fusion protein and cleavage products were separated by SDS-PAGE and quantified via optical densitometry. Based on the percentage of uncleaved substrate, the compound activity

was expressed as percentage ATG4B inhibition, which ranged from 3 to 82% depending on the benzotropolone compound applied in the assay (Figure 2).

3.1.2 *Benzotropolone compounds can inhibit autophagy in cells*

To investigate autophagy inhibition in a cellular setting, 18 benzotropolone compounds showing promising results in the LC3-GST cleavage test were subjected to a fluorescent CYTO-ID assay. This approach, which is based on selective (fluorescent) labeling of autophagosomes in live cells, enables rapid and quantitative monitoring of autophagy modulation via flow cytometry. Autophagy was stimulated in Jurkat T-cells by addition of 10 μ M mTOR inhibitor (everolimus) in the presence or absence of 10 μ M test compound. The CYTO-ID fluorescence readouts showed that seven benzotropolone compounds significantly inhibited everolimus-induced autophagosome formation (Figure 3). To confirm inhibition of autophagy, the assay was repeated in the presence of bafilomycin A1, a drug that prevents autophagy-dependent degradation of the protein markers LC3-II and SQSTM1. According to western blotting analysis, all benzotropolone structures that inhibited everolimus-induced CYTO-ID labeling yielded decreased LC3B-II and SQSTM1 levels indicative of autophagy inhibition (Figure 3).

3.1.3 *Benzotropolone compounds can inhibit ATG4B activity in cells*

Compounds showing significant activity in the CYTO-ID assay were further examined for ATG4B inhibition in HEK-293T cells transiently transfected with a plasmid encoding a β -actine-LC3-dNGLUC fusion protein. Active ATG4B enzyme will cleave the peptide bond between LC3 and dNGLUC, a truncated form of *Gaussia* luciferase (GLUC), thereby releasing dNGLUC into the culture medium where it is responsible for the production of a chemiluminescent signal upon addition of the luciferase substrate coelenterazine.(42) In the presence of an ATG4B inhibitor, cleavage of the fusion protein can be suppressed, resulting in

a decreased intensity of the observed signal. Hence, this method allows for an indirect though specific detection of intracellular ATG4B inhibition. Transfected HEK-293T cells were incubated for 6 hours with 10 μ M everolimus to stimulate autophagy or with a combination of 10 μ M everolimus and 10 μ M test compound. The increase in luciferase activity in everolimus-treated cells was efficiently blocked in the presence of all tested benzotropolone structures (Figure 4). To exclude the possibility that the test compounds blocked everolimus-induced inhibition of mTOR, a western blot for phosphorylated p70S6 kinase (p70S6K) was performed to assess mTOR activity. Everolimus was able to potently inhibit phosphorylation of p70S6K both in the absence and presence of benzotropolone compounds (Figure 4).

3.1.4 *Metabolization and stability of benzotropolone compounds*

Three compounds (UAMC-2386, UAMC-2500 and UAMC-2526) were selected for assessment of their stability in the presence of liver microsomes or plasma. Compound UAMC-2500 proved too unstable in the presence of microsomal enzymes (97% metabolized after 15 minutes) and hence was excluded from further tests. Compounds UAMC-2386 and UAMC-2526 remained moderately stable (45% and 70% metabolization, respectively, after 30 minutes), though compound UAMC-2386 had a very short plasma half-life (16 ± 1 minutes). Compound UAMC-2526 was fairly stable in plasma with a half-life of 126 ± 25 minutes and was chosen for further *in vivo* testing.

To ensure a continuous and constant administration of compound UAMC-2526 *in vivo*, we decided to use an osmotic minipump system that requires good solubility of the compound in the selected vehicle. Indeed, when solubility is compromised, the system can become dysfunctional due to precipitate formation, causing inconsistent product release. A turbidimetric solubility assessment of compound UAMC-2526 was performed at a concentration range of 10-80 mM in a vehicle that is compatible with the minipump (48).

Compared to blank measurements, no increase in turbidity was observed over a 2 hours period. Moreover, because the drug is loaded into a reservoir that is subcutaneously implanted, stability of the solubilized drug at body temperature for the entire duration of the treatment must be ensured. Therefore, a 40 mM stock solution of compound UAMC-2526 was incubated in vehicle at 37°C for a period of 31 days. Samples were taken at different time points and analyzed by LC-MS/MS. No compound degradation was observed over the course of 31 days (data not shown). Stability at 37°C was also investigated by following the activity of the compound *in vitro*. Samples of a 40 mM stock solution incubated at 37°C were taken at days 1, 23 and 31. Compound activity was tested in Jurkat T-cells and monitored by the CYTO-ID reagent as described above. No loss of compound activity was observed (Figure 5).

3.1.5 UAMC-2526 inhibits proliferation of HT-29 colorectal carcinoma cells

UAMC-2526 was added to HT-29 human colorectal carcinoma cells and cell proliferation was measured with WST-1 reagent. UAMC-2526 inhibited HT-29 proliferation in a concentration-dependent way (Figure 6A). Combined treatment of HT-29 cells with UAMC-2526 (50-100 μ M) and the antitumoral drug oxaliplatin (10 μ M) almost completely prevented cell proliferation (Figure 6B).

3.2 *In vivo* experiments

3.2.1 UMAC-256 inhibits autophagy in a healthy mouse model

The ability of test compound UAMC-2526 to suppress autophagy *in vivo* was first investigated in a healthy mouse model. The drug was administered to transgenic GFP-LC3 mice via a subcutaneously implanted osmotic minipump at a rate of 2 mg/kg/day. After 24 hours of drug administration, mice were starved overnight to induce autophagy and were subsequently sacrificed. Liver samples were taken for western blotting, immunohistochemistry and transmission electron microscopy. Animals treated with compound UAMC-2526 showed lower

levels of uncleaved (free) GFP, decreased LC3-II/LC3-I ratios and a marked increase of SQSTM1 as compared to control animals, indicating autophagy inhibition (Figure 7A). Moreover, microscopic analysis of formalin-fixed liver samples revealed a significant reduction of LC3 immunoreactive puncta (Figure 7B, upper panels) and GFP fluorescent dots (Figure 7B, lower panels), suggesting impaired autophagosome formation. Images obtained from transmission electron microscopy showed little or no autophagic vacuoles in the treated group vs. abundant vacuoles in the untreated control group (Figure 7C).

3.2.2 UMAC-2526 inhibits autophagy in a colorectal cancer xenograft model and potentiates chemotherapy efficacy

To determine the influence of autophagy inhibition on tumor growth and sensitivity to chemotherapy, HT-29 human colorectal carcinoma cells were injected in immunosuppressed CD1^{-/-} Foxn1^{nu} mice. After 10 days, mice developed tumors with a volume of approximately 200 mm³ and were randomly divided into 4 treatment regimens: vehicle, chemotherapy (oxaliplatin), autophagy inhibitor (UMAC-2526) or a combination of oxaliplatin and compound UMAC-2526. Clinical symptoms of toxicity such as a decrease in body weight were not observed during treatment (Figure 8A). Although the initial tumor volumes were not statistically different between the different treatment groups, the mean relative tumor volume (RTV) increased rapidly in control mice between D18 and D29 (Figure 8B). Accordingly, most control mice were sacrificed between D27 and D29 when tumors reached a volume of 1500 mm³. Compared to control, monotherapies showed a reduced RTV, but only the combination therapy led to a significant decrease in RTV from D24 onward (Figure 8B). Tumor progression tended to decrease after treatment with oxaliplatin (tumor growth delay: 3.1 days) or autophagy inhibitor (tumor growth delay: 2.2 days). However, only combined treatment led to a significant

growth delay of 4.6 days ($p < 0.05$). At D29 (19 days of treatment), administration of oxaliplatin or compound UAMC-2526 led to a tumor growth inhibition (TGI) of 51% and 44%, respectively, while combined treatment led to a TGI of 75%.

Microscopic analysis of H&E stained tumor sections revealed moderately differentiated to undifferentiated tumors, with viable solid sheets of tumor cells, typical glandular avascular regions and clearly delineated subcutis margins. In some tumors treated with the autophagy inhibitor, a more differentiated phenotype was observed, with tumor cells organized in distinct islands, surrounded by more desmoplastic stroma and the presence of more avascular glands (Figure 9A). The tumors were characterized by a high extent of necrosis (>30% of total area). However, necrosis levels were not different among the treatment groups (Figure 9A). Also the frequency of apoptosis did not change in treated animals versus controls (Figure 9B). The cell proliferation marker Ki67 was reduced in all treatment groups as compared to the control group, and the highest reduction was observed in the combination group (Figure 9C).

Tumor samples were processed for western blotting to analyse autophagy markers. Administration of compound UAMC-2526 decreased basal autophagy as measured by the ratio of LC3-II/LC3-I and SQSTM1 expression (Figure 10). Oxaliplatin treatment, however, led to induction of autophagy, which was blunted in the presence of UAMC-2526 (Figure 10). Compared to LC3-II, the effect of UAMC-2526 on SQSTM1 was less clear. This could be explained by the multifunctional role of SQSTM1 protein in cancer progression and the fact that it is not only involved in autophagy.(49–51) Inhibition of autophagy by UAMC-2526 was confirmed by electron microscopy showing a significant decrease in the formation of autophagic vacuoles in tumors of UAMC-2526 treated mice (Figure 11).

4. Discussion

Autophagy is an important cellular defense mechanism that is indispensable for maintaining cellular homeostasis in different stress situations.(10) Hence, autophagy might function in a tumor protective manner by relieving cellular stress such as hypoxia and nutrient deprivation and removal of damaged organelles, as is the case in fast growing tumors.(5) This assumption was confirmed in recent studies showing that genetic or pharmacological inhibition of autophagy enhances the cytotoxicity of chemotherapeutics and promotes tumor regression.(19–21) Accordingly, targeting autophagy in advanced tumors can be interesting as add-on therapy to existing anticancer therapies. It should be noted that ATG4B is a core autophagy related protein responsible for the conversion of inactive pro-LC3B to LC3B-I, which after lipidation by other ATG enzymes allows elongation of the autophagosomal membrane.(33,34) Because inhibition of this event will abolish autophagosome formation and thus impairment of autophagy, several research tools have been developed to identify compounds with ATG4B inhibiting abilities.(41,42,44) Recently, we reported that compounds with a benzotropolone backbone structure have ATG4B inhibiting potential.(44) The aim of the present study was the synthesis of a library of small molecules with a benzotropolone core and the assessment of their therapeutic value in cancer treatment.

Compounds were first screened in a gel-based assay for their ability to inhibit purified ATG4B activity on a LC3-GST substrate. Promising hit compounds were further tested *in vitro* using several cellular assays to confirm inhibition of intracellular ATG4B and impairment of autophagy. Because compound UAMC-2526 showed fair stability in mouse plasma, this molecule was tested in healthy, nutrient deprived GFP-LC3 mice. Nutrient deprivation is a simple yet robust method to induce autophagy in different tissues, particularly in the liver, which reacts very strongly to this stimulus.(45) Autophagy induction can be easily detected in GFP-LC3 mice by measuring cleavage of GFP-LC3 or by visualizing GFP fluorescent and/or

LC3 immunoreactive dots.(45,47) Liver samples of animals treated with compound UAMC-2526 showed lower levels of uncleaved (free) GFP, decreased LC3-II/LC3-I ratios, a marked increase of the autophagy scaffold protein SQSTM1 as well as a significant reduction of LC3 immunoreactive puncta and GFP fluorescent dots, indicating that UAMC-2526 impairs autophagosome formation and inhibits starvation-induced autophagy *in vivo*.

Because autophagy plays an essential role in tumor progression, the next step was to assess the suitability of autophagy inhibition via UAMC-2526 in cancer treatment. For this purpose, we used a tumor xenograft mouse model bearing subcutaneous HT29 colorectal cancer. Mice received either vehicle, UAMC-2526, the standard chemotherapy drug oxaliplatin or a combination of UAMC-2526 and oxaliplatin. Interestingly, administration of oxaliplatin strongly induced autophagy in tumor tissue as compared to vehicle-treated controls. This effect was largely prevented by co-treatment with UAMC-2526. Although both UAMC-2526 and oxaliplatin inhibited tumor growth, only combined therapy led to a significant reduction in relative tumor volume as compared to controls. Combined treatment also led to a significant tumor growth delay of 4.6 days and to 75% tumor growth inhibition (TGI), indicating that ATG4B inhibitors are effective as add-on treatment during chemotherapy. This effect was accompanied with a significant decrease in cell proliferation, which can be expected following treatment with autophagy blockers (52,53), and a weak induction of apoptosis. Nevertheless, these mechanisms may be inadequate to explain the high rate of TGI. Probably, also other effects are involved. Indeed, a remarkable feature in some tumors treated with UAMC-2526 was their more differentiated phenotype i.e., tumor cells organized in distinct islands that were surrounded by more desmoplastic stroma. This might indicate a less malignant phenotype, as more differentiated tumors (low grade) tend to grow and spread at a slower rate than poorly or undifferentiated tumors (high grade).(54) However, the role of autophagy inhibition in this process is still unclear. Our results are in line with a study of Selvakumaran et al. showing that

oxaliplatin induces autophagy in HT29 xenografts and that addition of chloroquine to oxaliplatin provides a better tumor control.(20) In both studies, inhibition of autophagy sensitized cancer cells to chemotherapy and augmented the cytotoxicity of the chemotherapeutic agent oxaliplatin as combined therapy decreased tumor cell proliferation and increased tumor apoptosis. Other small molecules have previously been reported by Akin et al. to inhibit ATG4B and to impair growth of osteosarcoma tumors *in vivo*.(37) Combined with our results, these findings show that autophagy inhibition via ATG4B could be a valuable strategy in treating different tumor types.

Importantly, several study limitations should be addressed. First, we administered only one dose to tumor bearing mice and acknowledge that the results could even have been improved if a higher dose was used. Toxicity should be minimal since benzotropolone derivatives are natural components of theaflavins found in green or black tea.(55) However, we were limited by the maximal solubility of the compound within the osmotic minipump. Second, ATG4B has been shown to promote colorectal tumor growth independent of autophagy via upregulation of mTOR signaling and cyclin D1 expression.(56) Therefore, despite clear blunting of oxaliplatin-mediated autophagy induction, other effects related to ATG4B inhibition could have contributed to the findings observed in the present study. Altogether, our results show that ATG4B inhibition and subsequent impairment of autophagy by small molecules with a benzotropolone backbone structure is an effective approach to augment the sensitivity to chemotherapy and to attenuate tumor growth.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

List of abbreviations

3MA, 3-methyladenine; ACTB, actin, beta; ATG4B, autophagy related 4B cysteine peptidase; GFP, green fluorescent protein; LC3B, microtubule-associated protein light chain 3B; GST, glutathione-S-transferase; mTOR, mechanistic target of rapamycin; p70S6K, ribosomal protein S6 kinase B1; SQSTM1, sequestosome 1.

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Figure legends

Figure 1. Benzotropolone synthesis.

A benzotropolone structure in which R4-6 = H can be obtained by mixing the catechol derivative S-1 and the gallic acid derivative S-2 and adding potassium iodate to obtain the benzotropolone S-3. Methylation of R4-6 can then be achieved by adding anhydrous DMF, sodium hydroxide and iodomethane.

Figure 2. ATG4B inhibition assay.

Compounds with a benzotropolone backbone structure were added to a test tube containing recombinant LC3-GST substrate and ATG4B enzyme for 6 minutes at room temperature after which the reaction was stopped by addition of 10 μ L of Laemmli buffer. Samples were then loaded on NuPAGE gels and subjected to SDS-PAGE. Cleavage products were quantified and inhibition was calculated as described in the methods section, results are obtained from three independent experiments.

Figure 3. *In vitro* autophagy inhibition assay.

Jurkat T-cells were treated for 16 hours with 10 μ M everolimus to induce autophagy in the presence or absence of 10 μ M test compound. Cells were then washed and stained with CYTO-ID autophagy detection reagent for 30 minutes and analyzed by flow cytometry (upper panel). Alternatively, 10 μ M bafilomycin A1 was added to inhibit autophagic flux and cells were subjected to western blotting for the autophagy markers LC3 and SQSTM1 (lower panel). 3-methyladenine (3MA) was used as a positive control for autophagy inhibition. Data were analyzed using One Way ANOVA followed by Bonferroni's post-hoc test. Four independent experiments were performed, *** $P < 0.001$, significantly different from everolimus treated cells.

Figure 4. *In vitro* ATG4B inhibition assay.

HEK 239T cells were transfected with pEAK12 plasmid DNA encoding truncated *Gaussia* luciferase (dNGLUC) fused to β -actine and LC3 (β -actine-LC3-dNGLUC). After 24 hours, cells were treated for 6 hours with 10 μ M everolimus to induce autophagy in the presence or absence of 10 μ M test compound. Luciferase activity was measured in the medium using a BioLux *Gaussia* luciferase assay kit (upper panel). Downstream mTOR target phospho-p70S6k was analyzed in cell lysates via western blotting to rule out mitigation of everolimus-induced mTOR inhibition. Data were analyzed using One Way ANOVA followed by Bonferroni's post-hoc test. Four independent experiments were performed, $**P < 0.01$; $***P < 0.001$, significantly different from everolimus treated cells, $\#P < 0.05$; $###P < 0.001$, significantly different from untreated control.

Figure 5. Stability of UAMC-2526 at 37°C.

Compound UAMC-2526 (10 mM) was dissolved in a vehicle composed of DMSO, propylene glycol, ethanol (50:40:10) and supplemented with 0.4 μ L/mL Tween 20 and stored in an incubator at 37°C. At different time points, *in vitro* autophagy inhibition was measured by flow cytometry as shown in Figure 3. Data were analyzed using Two Way ANOVA followed by Bonferroni's post-hoc test. Four independent experiments were performed, $***P < 0.001$, significantly different from everolimus treated cells.

Figure 6. Inhibition of HT-29 colorectal carcinoma cell proliferation by UAMC-2526 with or without oxaliplatin.

(A) HT-29 colorectal carcinoma cells were treated with different concentrations of UAMC-2526. Cell proliferation was measured at different time points with WST-1 proliferation reagent. (B) The experiment described in panel A was repeated in the presence of the anti-

tumoral drug oxaliplatin (10 μ M). Data were analyzed using Two Way ANOVA followed by Bonferroni's post-hoc test. Four independent experiments were performed, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, significantly different from untreated control.

Figure 7. Analysis of autophagy inhibition in nutrient-deprived GFP-LC3 mice.

Compound UAMC-2526 (or vehicle) was administered to transgenic GFP-LC3 mice at a constant rate of 2 mg/kg/day via subcutaneously implanted osmotic minipumps. After 24 hours, animals were starved overnight to induce autophagy. Mice were given an I.P. injection of 100 mg/kg chloroquine to inhibit autophagic flux 3 hours before sacrifice. Liver samples were collected. (A) Western blotting was performed to assess the ratio of free GFP/GFP-LC3, LC3-II/LC3-I and accumulation of SQSTM1. β -actin served as a loading control. Representative blots are shown. (B) Liver samples were immunohistochemically stained for LC3 (upper panel) or analyzed with fluorescence microscopy for the appearance of GFP positive dots (lower panel). Scale bar = 100 μ m. (C) Autophagic vacuoles (black arrows) were detected in ultrathin sections using TEM. Scale bar = 1 μ m. All data were analyzed with Student's t-test, $n = 7$ mice for both groups, $**P < 0.01$; $***P < 0.001$, significantly different from control animals.

Figure 8. Effect of autophagy inhibition on HT-29 tumor growth in CD1^{-/-} Foxn1nu mice.

(A) Ten days after tumor inoculation (D10), tumor-bearing mice were treated with vehicle (control), UAMC-2526, oxaliplatin or a combination of UAMC-2526 and oxaliplatin. Treatments had no effect on body weight. (B) Relative tumor volumes were calculated. Effects of treatment on RTV values were examined with general linear mixed models, including time, treatment and their interaction as fixed effects. Significant values of post hoc comparisons were adjusted using a step-down Bonferroni correction, $n = 11$ mice for all groups, $*P < 0.05$, significantly different from control animals.

Figure 9. Histological analysis of HT29 colorectal tumors.

After treatment of HT29-bearing CD1^{-/-} Foxn1^{nu} mice with vehicle (control), UAMC-2526, oxaliplatin or a combination of UAMC-2526 and oxaliplatin, HT29 tissue samples were stained with (A) hematoxylin-eosin or (B) immunostained for Ki67 or (C) cleaved caspase 3. Arrow heads indicate areas of more pronounced tissue differentiation. Scale bar = 500µm. Data were analyzed using Two Way ANOVA followed by Bonferroni's post-hoc test, $n = 11$ mice for all groups, $**P < 0.01$; $***P < 0.001$, significantly different from control animals, $^{\#}P < 0.05$ significant different from oxaliplatin-treated animals.

Figure 10. Intratumoral autophagy inhibition.

HT29 tissue samples were homogenized and subjected to western blotting to assess the ratio of LC3-II/LC3-I and SQSTM1 expression. β -actin served as a loading control. Representative images are shown. Representative blots are shown. Data were analyzed using Two Way ANOVA followed by Bonferroni's post-hoc test, $n = 11$ mice for all groups, $*P < 0.05$; $***P < 0.001$, significantly different from control animals.

Figure 11. Electron microscopy confirms intratumoral autophagy inhibition.

Tumor ultrathin sections were analyzed for the presence of autophagic vacuoles (black arrows) using TEM. Representative images are shown. Scale bar = 1µm. Data were analyzed using Two Way ANOVA followed by Bonferroni's post-hoc test, $n = 11$ mice for all groups, $*P < 0.05$; $***P < 0.001$, significantly different from control animals.

Tables

Table 1. Derivatization of benzotropolones

UAMC COMPOUND	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶
1356	-COOMe	-H	-H	-H	-H	-H
1409	-COOH	-H	-H	-H	-H	-H
2153	-CONH ₂	-H	-H	-H	-H	-H
2384	-CONHCH(CH ₃) ₂	-H	-H	-H	-H	-H
2385	-CONHCH ₂ CH(CH ₃) ₂	-H	-H	-H	-H	-H
2386	-CO-piperidine	-H	-H	-H	-H	-H
2387	-CONH-phenyl	-H	-H	-H	-H	-H
2388	-CONH-benzyl	-H	-H	-H	-H	-H
2449	-CO-tbocpiperazine	-H	-H	-H	-H	-H
2450	-CO-piperazine	-H	-H	-H	-H	-H
2507	-H	-H	-OMe	-Me	-Me	-Me
2508	-H	-H	-OMe	-Me	-H	-Me
2471	-COOH	-H	-OMe	-H	-H	-H
2472	-CONH(CH ₂) ₂ -4-fluorophenyl	-H	-H	-H	-H	-H
2473	-CONH-phenyl	-H	-F	-H	-H	-H
2477	-COOH	-Cl	-H	-H	-H	-H
2500	-COO(CH ₂) ₃ CH ₃	-H	-H	-H	-H	-H
2478	-CONHCH ₃	-H	-OMe	-H	-H	-H
2479	-CON(CH ₃) ₂	-H	-F	-H	-H	-H
2480	-CONH ₂	-H	-F	-H	-H	-H
2481	-CONH ₂	-H	-OMe	-H	-H	-H
2474	-CONHCH(CH ₃) ₂	-H	-Br	-H	-H	-H
2501	-COOH	-H	-Br	-H	-H	-H
2502	-COO(CH ₂) ₂ CH ₃	-H	-H	-H	-H	-H
2525	-COO(CH ₂) ₃ CH ₃	-Br	-H	-H	-H	-H
2526	-COO(CH ₂) ₃ CH ₃	-H	-Br	-H	-H	-H
2676	-COOH	-H	-(CH ₂) ₂ NH ₂	-H	-H	-H

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 8

Figure 10

Figure 11