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Original Article

Tubulin-binding anticancer polysulfides induce cell death via mitotic arrest and autophagic interference in colorectal cancer

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ABSTRACT

Polysulfanes show chemopreventive effects against gastrointestinal tumors. We identified diallyl tetrasulfide and its derivative, dibenzyl tetrasulfide (DBTTS), to be mitotic inhibitors and apoptosis inducers. Here, we translate their application in colorectal cancer (CRC). MALDI-TOF-MS analysis identified both compounds as reversible tubulin binders, validated by *in cellulo* α-tubulin degradation. BRAF(V600E)-mutated HT-29 cells were resistant to DBTTS, as evidenced by mitotic arrest for 48 h prior to apoptosis induction compared to KRAS(G12V)-mutated SW480/620 cells, which committed to death earlier. The prolonged mitotic block correlated with autophagy impairment and p62 protein accumulation in HT-29 but not in SW480/620 cells, whereas siRNA-mediated p62 inhibition sensitized HT-29 cells to death. *In silico* analysis with 484 colorectal cancer patients associated higher p62 expression and reduced autophagic flux with greater overall survival. Accordingly, we hypothesized that DBTTS targets CRC survival/death through autophagy interference in cell types with differential autophagic capacities. We confirmed the therapeutic potential of DBTTS by the inhibition of spheroid and colony formation capacities in CRC cells, as well as in HT-29 zebrafish xenografts *in vivo*.

Introduction

Despite progress in molecular testing and identification of predictive biomarkers, colorectal cancer (CRC) remains the second leading cause of cancer-related deaths worldwide. Chemotherapy is associated with limited efficacy in tumors at stages ≥ II. KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase), and BRAF (v-Raf murine sarcoma viral oncogene homolog B) are among the most frequently mutated oncogenes [1,2]. Testing for KRAS/BRAF status is a criterion for prognostic stratification, used primarily as an unfavorable marker to preclude specific therapies (e.g., anti-epithelial growth factor receptor [EGFR] therapy). Additionally, a high percentage of patients with one or both of these mutations do not respond to all major drug combination regimens, including those involving monoclonal antibodies. The reasons behind this lack of response are still unclear in most cases.

Abbreviations: Baf, bafilomycin A1; BRAF, v-Raf murine sarcoma viral oncogene homolog B; CFAs, colony formation assays; CHCA, α-cyano-4-hydroxy-cinnamic acid; CHX, cycloheximide; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CRC, colorectal cancer; DATTS, diallyl tetrasulfide; DBTTS, dibenzyl tetrasulfide; DHB, 2,5-dihydroxybenzoic acid; EGFR, epithelial growth factor receptor; FDR, false discovery rate; GO, gene ontology; HO, heme-oxygenase; KEAP1, kelch-like ECH-associated protein 1; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LC3, microtubule-associated protein light chain 3; MALDI-TOF-MS, matrix assisted laser desorption ionization-time of flight-mass spectrometry; MSI, microsatellite instability; MTA, microtubule-altering agent; mTOR, mammalian target of rapamycin; NRF2, nuclear factor (erythroid-derived 2)-like 2; NF-κB, nuclear factor-kappa B; OSC, organic sulfur compound; PGP, p-glycoprotein; PI, propidium iodide; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homolog; Q-RT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SD, standard deviation; siRNA, small inhibitor RNA; TEM, transmission electron microscopy; TFA, trifluoroacetic acid; TP53, tumor protein 53; TREAT, t-tests relative to a threshold.

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Many epidemiological studies have confirmed the chemopreventive effect of a diet that includes onions and garlic. Subsequent experimental studies have identified organic sulfur compounds (OSCs), specifically polysulfanes, as potential targets for anticancer drug development [3]. Tumors of the gastrointestinal tract could potentially benefit from the chemopreventive effect of OSCs. We previously showed that garlic-derived OSCs targeted the microtubular network and inhibited tubulin polymerization, leading to mitotic arrest [4]. Benzyl tetrasulfide (DBTTS), a derivative of the natural compound diallyl tetrasulfide (DATTS), has demonstrated improved pharmacological and anti-cancer properties compared to its naturally occurring counterpart [5]. Moreover, DBTTS diffuses and crosses membrane barriers without requiring specific transport systems for internalization or extrusion [6].

Blocking mitotic progression by targeting microtubules of proliferating tumor cells is a consolidated strategy, which relies on prototypical natural agents such as *Vinca* alkaloids (*Vinca rosea*) or taxanes (*Taxus brevifolia*) [7] and more recently, epothilones (i.e., *Sporangium cellulosum*) [8]. Mitotic inhibitors are widely used in combination treatments for various types of lung, bladder, and ovarian tumors. However, they are currently not in clinical use to treat colon cancer, a disease that exhibits a high level of chemoresistance towards spindle poisons, as taxanes have failed to provide significant clinical benefits in phase II trials [9]. P-glycoproteins (PGPs) are highly expressed in the gastrointestinal tract and contribute to resistance to paclitaxel [10].

Increased rates of autophagy are a hallmark of colorectal cancer. The most frequently administered 5-fluorouracil promotes autophagy, and strategies that involve impairing the autophagic flux can sensitize colon cancer cells to death [11]. As the modulation of the microtubular network impacts progression of the autophagic flux, novel compounds interfering with the autophagic flux in CRC could lead to therapeutic improvement.

In this study, the tubulin binder DBTTS triggered mitotic arrest followed by apoptosis in both KRAS(G12V)-mutated SW480/SW620 and BRAF(V600E)-mutated HT-29 cells. In HT-29 cells, stalled autophagy was concomitant with the accumulation of the p62 protein in mitotically arrested cells. Conversely, siRNA-mediated targeting of p62 sensitized cells to apoptosis. Autophagosomes also accumulated in DBTTS-treated SW480/SW620 cells concomitant with apoptosis induction. We validated the anti-cancer potential of DBTTS in 3D spheroid and colony formation assays, which was further confirmed by *in vivo* inhibition of HT-29 xenograft growth in zebrafish. Altogether, our results provide evidence of the *in vitro* and *in vivo* capacity of DBTTS to efficiently target CRC, pointing to the existence of a cell-type dependent autophagy impairment involving p62.

Materials and methods

Cell culture and reagents

The colorectal cancer cell lines HT-29 (Deutsch Sammlung von Microorganismen und Zellkulturen, DSMZ, Leibniz, Germany), SW480, and SW620 (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, Bornem, Belgium) and 1% (v/v) antibiotic-antimycotic (BioWhittaker). Cells were seeded at a density of 3×10^5 cells/ml in 6-well plates to achieve a confluence of 60% at the moment of treatment, after a 24 h incubation in a humidified atmosphere at 37 °C and 5% CO_2.

DATTS and DBTTS were synthetized, as previously described [5], with DBTTS showing improved solubility in aqueous media, reduced volatility and odor, powdery consistency, and suitable lipophilicity to cross membranes by Lipinski's Rule of Five [5]. DATTS and DBTTS were dissolved in dimethyl sulfoxide (DMSO; stock solution: 50 mM). Vinblastine, vincristine (stock solutions: 1.2 mM), and paclitaxel (stock solution: 7 mM), were obtained from Teva® Pharmaceutical Industries (Petach Tikva, Israel) as ready-to-use injectable solutions; intermediate stocks were freshly prepared and dissolved in sterile water. The vacuolar pump inhibitor bafilomycin A1 (Baf; 10 nM, stock solution: 100 μM) and the mammalian target of rapamycin (mTOR) inhibitor PP242 (10 μM, stock solution: 10 mM) were obtained from Sigma-Aldrich. The protein synthesis inhibitor cycloheximide (CHX; 10 μM, stock solution: 10 mM) and the proteasome inhibitor MG132 (5 μM, stock solution: 50 mM) were purchased from Calbiochem (Leuven, Belgium). All compounds were dissolved in DMSO.

Cell viability measurement and apoptosis assessment

Cell viability was measured using 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reagent (Sigma-Aldrich) according to the manufacturer's instructions. Apoptosis was assessed through morphological analysis of the nucleus following Hoechst 33342 staining (1 μg/ml, 15 min; Sigma-Aldrich) using fluorescence microscopy (Olympus, Aartselaar, Belgium) [12].

Cell cycle assessment

Cell cycle was analyzed according to standard procedures. Briefly, adherent cells were trypsinized, and both floating and trypsinized cells were harvested. After centrifugation (5 min, 1200 rpm), pellets were fixed with 70% (v/v) ethanol for at least 40 min on ice. Cells were incubated for 20 min in phosphate buffer solution (PBS) containing one μg/ml of propidium iodide (PI; Becton Dickinson Biosciences, Erembodegem, Belgium) and 100 μg/ml RNase A (Roche, Luxembourg). Flow cytometry (FACScalibur, BD Biosciences, San José, CA, USA), with the CellQuest software (BD Biosciences, Erembodegem, Belgium), was used to record 10,000 events/sample. Data were analyzed with the FlowJo887 software (Tree Star Inc., Ashland, OR, USA).

Caspase-3-7 assay

The caspases-3/-7 activity assay was assessed using Caspase-3/-7® Glo assay purchased from Promega (Leiden, The Netherlands). Cells (2.0×10^3) treated with/without 50 μM of DBTTS were incubated for 1 h at room temperature with 50 μl of reagent. Caspase activity was monitored using a Centro LB 960 plate-reading luminometer (Berthold, Bad Wildbad, Germany).

Matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis

Chemicals and reagents. All solvents were high-performance liquid chromatography (HPLC) grade. Acetonitrile was obtained from Thermo Fisher Scientific (Geel, Belgium), and 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxy-cinnamic acid (CHCA) were obtained from Sigma-Aldrich (St Quentin-Fallavier, France). Trifluoroacetic acid (TFA) was purchased from Merck Schuchardt (Hohenbrunn, Germany). HTS tubulin (purity of 97%, Cat #HTS02-B/092) and the tubulin buffer (Cat #BST01-001/069) were purchased from Cytoskeleton (Boechout, Belgium).

Matrices. Different matrices and solvent conditions were evaluated to analyze DATTS and DBTTS using MALDI-TOF-MS. These conditions are listed in Table 1. All depositions were made using the dried-droplet method with an analyte/matrix ratio of 1 to 1.

MALDI-TOF-MS. MALDI-TOF-MS measurements were carried out on a Bruker ultraflex II TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with the SCOUT 384 probe ion source, using a nitrogen pulsed laser (337 nm, model VSD-337ND; Laser Science Inc., Boston, MA, USA) with an output energy of 400 μJ/pulse. The ions were accelerated without delayed extraction conditions in positive ion mass reflectron mode with ion source 1 (IS1) set to 25.13 kV, ion source 2 set to 21.18 kV (84.4% IS1), the lens set to 9.05 kV (36% IS1), the reflector set to 26.43 kV (13.87 kV [52.5% reflection]), using the "RP_0-1kDa.par" method. All spectra data presented resulted from four series of 200 laser shots from four different locations ($[M+H]^+$ for the different species). The positive ions were accelerated with a 200-ns extraction delay. The source pressure was 10^{-6} mbar, and the analyzer pressure was 10^{-7} mbar. Mass spectra were manually acquired, using FlexControl (Bruker Daltonik GmbH) software, by accumulating four series of 1000 laser shots, and the respective analysis was performed using the FlexAnalysis software. Internal calibration was conducted using DHB or CHCA matrix peaks, depending on the matrix used. The DHB peaks used were: the DHB monoisotopic peak, *m/z* 154.02606 ($M^{\bullet+}$), *m/z* 155.03389 ($[M+H]^+$), *m/z* 273.03936 ($[2M-2H2O]^+$), *m/z* 137.0233 ($[M-OH]^+$), *m/z* 308.0532 ($[2M]^{\bullet+}$), and values of DHB adducts with ions such as Na^+ (*m/z* 177.0158) and K^+ (*m/z* 192.9898). The CHCA

Table 1
Development and optimization of the MALDI-TOF-MS conditions. Observed ions in MALDI-TOF-MS for DATTS and DBTTS are reported with related statistics for the incubated samples.

Compounds	Observed ions	Observed *m/z*	Theoretical *m/z*	Δm (ppm)
DATTS	$[M-H]^+$	208.9597	208.9587	4.8
	$M^{\bullet+}$	209.9653	209.9665	5.7
	$[M+H]^+$	210.9126	210.9685	−28
DBTTS	$[M-H]^+$	308.0051	308.9968	22
	$M^{\bullet+}$	310.0032	309.9973	−1.6
	$[M+H]^+$	311.0013	310.9877	−57