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Death Inducing and Cytoprotective Autophagy in T-47D Cells by Two Common Antibacterial Drugs: Sulfathiazole and Sulfacetamide

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Abstract

The broad spectrum of the pharmacological effects of sulfonamide family of drugs motivated us to investigate the cellular mechanisms for anti-cancer effects of sulfathiazole and sulfacetamide on T-47D breast cancer cells. Fluorescent microscopy, flow cytometric analysis, caspase-3 activity and DNA fragmentation assays were used to detect apoptosis. The distribution of the cells among different phases of the cell cycle was measured by flow cytometry. The expression of several genes with important roles in some critical cellular pathways including apoptosis, mTOR/AKT pathway and autophagy were determined by real time RT-PCR analysis. Sulfathiazole and sulfacetamide induced anti-proliferative effects on T-47D cells were independent of apoptosis and cell cycle arrest. The overexpression of critical genes involved in autophagy including ATG5, p53 and DRAM indicated that the main effect of the drug-induced anti-proliferative effects was through induction of autophagy. This process was induced in 2 different forms, including death inducing and cytoprotective autophagy. Sulfathiazole treatment was followed by higher expression of p53/DRAM and downregulation of Akt/ mTOR pathway resulting in death autophagy. In contrast, sulfacetamide treatment lowered expression of p53/DRAM pathway in parallel with upregulation of Akt/mTOR pathway promoting cytoprotective autophagy. The results indicated that autophagy is the main mechanism mediating the anti-cancer effects of sulfathiazole and sulfacetamide on T-47D cells. Alignment of the p53 and DRAM expression along with activation level of Akt survival pathway therefore determines the type of autophagy that occurs.

Keywords

Sulfathiazole; Sulfacetamide; Breast cancer; Autophagy; p53/DRAM pathway; Apoptosis

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Introduction

Evins and Phillips synthesized sulfapyridine in 1937, which was the first sulfonamide used with great success in combating pneumonia (Gennaro, 1990). Sulfonamides compete with *p*-aminobenzoic acid to prevent its normal incorporation into folic acid (Seydel 1968; Supuran *et al.*, 2003). Thus, sulfa drugs inhibit growth and proliferation of bacteria that depend on the cellular synthesis of folic acid (Seydel 1968; Supuran *et al.*, 2003).

Of a large number of structurally novel sulfonamide derivatives, some have anti-cancer effects. Among them, E7070 and E7010 act by inhibiting the assembly of microtubules, resulting in cell cycle arrest (Owa *et al.*, 1999; Fukuoka *et al.*, 2001). Indole sulfonamides inhibit cancer cell proliferation during mitosis via inhibition of microtubular spindle assembly, and induce apoptosis in a bcl-2 dependent apoptotic pathway (Mohan *et al.*, 2006). J30, a synthesized sulfonamide, inhibits assembly of purified tubulin by strongly binding to the colchicine-binding site resulting in accumulation of cancer cells in G₂/M phase of the cell cycle (Liou *et al.*, 2007). J30 mediated apoptotic signaling pathway also depends on caspase activation and cytochrome c release (Liou *et al.*, 2007).

HMN-176, another synthetic sulfonamide, shows potent cytotoxicity toward various human tumor cells, and arrests cells in M phase through destruction of spindle polar bodies, followed by DNA fragmentation (Gottesman *et al.*, 1993). HMN-176 also can inhibit the expression of MDR1 at the transcriptional level by inhibiting NF- κ B activity in human ovarian cancer cells (Tanaka *et al.*, 2003). An aromatic sulfonamide derivative, E7820, inhibits proliferation and tube formation of human umbilical vein endothelial cell (HUVEC), and is a novel antiangiogenic sulfonamide derivative (Funahashi *et al.*, 2002).

Acetazolamide inhibits the invasiveness of renal carcinoma cells by carbonic anhydrase inhibition (Parkkila *et al.*, 2000). Thus, sulfonamide derivatives show anti-cancer effects through different cell specific mechanisms. Thus we have investigated the mechanisms of anti-proliferative action of 2 members of sulfonamide drug family, sulfathiazole and sulfacetamide, on T-47D breast cancer cells.

Sulfathiazole with chemical formula C₉H₉N₃O₂S₂ has antibacterial and antimicrobial activity. It is used as diuretic, a carbonic anhydrase inhibitor and an anti-convulsant (Liao, 2003). Sulfacetamide (sulfanilylacetamide) with chemical formula C₈H₁₀N₂O₃S has a common clinical application in combination with two other sulfa drugs sulfathiazole and sulfabenzamide for treatment of certain vaginal infections (Valley and Balmer, 1999). Thus far, no anti-cancer effects have been reported for sulfathiazole and sulfacetamide. We have mainly analyzed the anti-tumor action of sulfathiazole and sulfacetamide, and demonstrated that the induction of autophagy is a major cellular mechanism by which the 2 sulfadugs exert their onco-suppressive activity in T-47D cells, giving new insight into the distinction between cell survival and cell autophagy mechanisms.

Material and Methods

Materials

RPMI 1640 and fetal bovin serum (FBS) were from Gibco (England). Streptomycin and penicillin were from Roche (Germany). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Sodium salt of sulfathiazole and sulfacetamide were from Sina Darou-Iran and Doxorubicin was from Ebewe Pharma (Austria). Annexin-V-FLOUS Staining Kit, Propidium Iodide (PI) kit, DNA Laddering kit, caspase-3 fluorometric immunosorbent enzyme assay kit, 4',6-diamidino-2-phenylindole (DAPI) kit were obtained from Roche. RevertAid™ M-MuLV reverse transcription enzyme and random hexamer primer were from Fermentas company-Germany. Agarose was purchased from Promega-USA. GeneRuler Ladder Mix (100–1000 bp) was from Fermentas. Primers were prepared by TAG Copenhagen-Denmark. RNeasy Plus Mini kit and QuantiFast SYBR Green PCR kit were from Qiagen-USA.

Methods

Cell culture and treatments—T-47D cells (ATCC number HTB-133) were purchased from National Cell Bank of Pasteur Institute (Tehran, Iran). Cells were cultured in RPMI medium 1640, supplemented with 10% FBS and 1% penicillin/streptomycin, in a humidified atmosphere of 5% carbon dioxide in air at 37°C. According to MTT assay, the LC₅₀ of sulfathiazole and sulfacetamide after 48 h was determined as 6.5 mM and 41 mM, respectively. Doxorubicin and sodium salt of sulfadiazole were dissolved in culture medium to the final desired concentration based on the determined LC₅₀ and filtered. Cells (at 80% confluency) were incubated with freshly prepared drugs for 48h in a humidified incubator before being trypsinized and washed with phosphate-buffer saline 3 times and stored at –70°C.

Cytotoxicity/Viability Test—For cell viability assay, cells were seeded in at least triplicate wells for each concentration of drug per time at 1×10^4 cells/well in a 96-well plate. After 24h of seeding, the cells had grown to ~80% confluency. The medium was changed to that containing drugs at concentrations ranging from 0.0–50 mM. The concentration range for doxorubicin was 0–6 µM. After 24, 48 and 72 h, each well was filled with 25 µl MTT stock solution (4 mg/ml or 100 µg/well) and incubated for 3 h at 37°C. Formazan crystals were dissolved in 100 µl of dimethyl sulfoxide (DMSO) and quantified using a microplate reader (Rayto-China) at 570 nm. The MTT assays were performed at least 3 times for each drug and the percentage of surviving cells relative to control (untreated sample) was calculated.

Quantification of apoptosis and Cell cycle analysis by flow cytometry—To quantify drug-induced apoptotic cell death, Annexin V-FITC and propidium iodide (PI) staining was performed using Annexin-V-FLOUS and PI staining kit, followed by flow cytometry using a PartecPass instrument (USA). Cells were incubated with 6.5 mM and 41 mM of sulfathiazole and sulfacetamide, respectively, for 48 h. The cells (10^6) were washed in PBS and suspended in 100 µl Annexin/PI buffer (20 µl of each Annexin and PI buffer in 1 ml incubation buffer) for 10–15 min at 25°C. After dilution in 500 µl incubation buffer,

fluorescence was measured at excitation and emission wavelengths of 518 nm and 617 nm, respectively. Cell cycle distribution was determined using DAPI staining kit. Cells (5×10^5) cells were incubated with 1 ml fluorochrome solution (10 $\mu\text{g/ml}$, DAPI; and 6% triton X-100 in PBS) in the dark for 30 min at 4°C . Fluorescence was recorded at excitation and emission wavelengths of 359 nm and 461 nm, respectively, using the PartecPass flow cytometer. Data was analyzed on 2-dimensional curves (number of cells against area under the peak), using FloMax software.

Morphological assays by fluorescent microscopy: Briefly, cells were cultured on coverslips and incubated for 48 h with each drug at their LC_{50} values (6.5 mM and 41 mM for sulfathiazole and sulfacetamide, respectively). The medium was removed and the coverslips covered with Annexin-V-FITC staining solution (20 $\mu\text{l/ml}$ for Annexin-V-FITC and 20 $\mu\text{l/ml}$ of PI) for 10–15 min in the dark at room temperature. Following incubation, the coverslips were mounted and examined using a fluorescent microscope (Karl Zeiss-Germany) under excitation wavelength range of 450–500 nm and green detection range of 515–565 nm.

Caspase-3 activity assay—Caspase-3 activity was determined using a fluorometric immunosorbent enzyme assay kit. After washing, cells (2×10^6) with PBS, cells were lysed in buffer. The experiments were carried out in multi-well plates coated with 100 μl of anti-caspase-3 and incubated at 37°C for 1 h. The coating solution was removed and non-specific binding sites on the plate blocked by adding 200 μl blocking buffer. After removing this buffer, the plates were washed 3 times with incubation buffer; 100 μl of cell lysate was added to the wells, and incubated at 37°C . After 1h, lysates were removed by aspiration and the cells were washed 3 times with incubation buffer. Fresh substrate solution was added to each well, and fluorescence intensity was measured after 2 h at excitation and emission wavelengths of 400 nm and 505 nm, respectively.

DNA laddering assay—After drug treatment, DNA was extracted and purified using DNA laddering assay kit to evaluate fragmentation of DNA. The design of the kit is based on attachment of specific concentration of DNA molecules to glass fiber fleece in the presence of guanidine-HCL. Cells (2×10^6), both attached and floating cells, were lysed using binding/lysis buffer for 10 min at room temperature and DNA was extracted using a weak salt solution elution buffer (10 mM Tris, pH 8.5). DNA concentration was determined spectroscopically and 1–3 μg of purified DNA electrophoresed at 75V for 1.5 h using a 1% agarose gel in Tris-borate buffer and visualized by ethidium bromide staining. U937 cell treatment with 4 $\mu\text{g/ml}$ camptothecin for 3 h was used as positive control, which resulted in apoptosis of 30% of the cells.

Total RNA extraction, cDNA synthesis and real time RT-PCR analysis—Total RNA was extracted from treated cells (6.5 mM and 41 mM for sulfathiazole and sulfacetamide, respectively) using the RNeasy Plus Mini kit. First-strand cDNA was generated by RevertAidTM M-MuLV reverse transcriptase using 5 μg total RNA and random primers, with the following program: 25°C for 10 min, and 1 h at 42°C . To quantify the level of mRNA expression real time RT-PCR was performed using the QuantiFast

SYBR Green PCR Master Mix according to the following program: 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec, T_M for 25 sec and 72°C for 30 sec with melting curve carried out in a Corbett real-time PCR system. The data generated were analyzed by Corbett Software, using the comparative C_T method (or C_T method), and the relative amount of target material was quantified compared to the reference genes. Primers for real time T-PCR has been listed in Table 1 (available in Online Resource 1).

Statistical analysis

Statistical analysis used by the SPSS version 16 and Excel 2007 software. Flow cytometry, caspase-3 activity and real time RT-PCR were compared between each group and its control. Results are expressed as mean $\pm$ standard error of the mean of the values obtained in 3 separate experiments. Differences of $p < 0.05$ were considered statistically significant. * indicates significant difference at $p < 0.05$ and ** at $p < 0.01$.

Results

Sulfathiazole and sulfacetamide treatment caused time- and dose-dependent reduction in cell number

Cell metabolic activity was assessed by succinate dehydrogenase (SDH) activity, a measure of cellular mitochondrial respiration. MTT assay was carried out to ascertain the relative cell viability after incubation with different concentrations of drugs for 24, 48 and 72 h. This was achieved by drawing the absorbance plot of the formazan produced (dissolved in DMSO) for increasing drug concentration at each incubation time. The concentration range of the two drugs was adjusted to 0.0–50 mM. There was a 50% reduction in cell viability (LC_{50}) after 48 h at 6.5 mM and 41 mM for sulfathiazole and sulfacetamide, respectively (Figure 1). LC_{50} for doxorubicin (positive control for cell cycle arrest) was 0.33 μ M after 48 h (data not shown).

Absence of apoptosis in sulfathiazole and sulfacetamide treated cells

Direct observation of cells cultured on a coverslip and incubated with drugs for 48 h showed that apoptotic and necrotic cells could be rarely detected by double staining with Annexin V-PI. They were only observed when several different microscopic fields were evaluated simultaneously (Figure 2; available in Online Resources 2–5). Rounding of the cells as a result of breaking away of cell junctions in parallel with exposing the membranous phosphatidylserines stained with Annexin V-FITC resulted in visualization of apoptotic cells by fluorescence microscopy (Figure 2a, c and d; available in Online Resources 2, 4 and 5, respectively). The late stage apoptotic cells showed accumulation of PI in the nucleus, and exposure of the phosphatidylserines on the outer leaflet of the plasma membrane. They were seen as green fluorescent shining circles having red nucleus (Figure 2a, c; available in Online Resources 2, 4 and 5, respectively). Living cells (unstain) were not detectable in fluorescent visual field, but are were by phase contrast microscopy (not shown).

Similar results were observed by flow cytometric studies of T-47D cells incubated with the drugs. Figures 3A and B show after dividing the 2 dimensional plots into 4 quadrants of Q_1 , Q_2 , Q_3 and Q_4 representing necrotic cells (Anx^- and PI^+), late apoptotic cells (Anx^+ and

PI⁺), normal cells (Anx⁻ and PI⁻) and early apoptotic cells (Anx⁺ and PI⁻), respectively. The vast majority of cells were in Q₃ region (Figure 3A and B). Hence, flow cytometry data supported the lack of apoptosis or necrosis in T-47D cells incubated with sulfathiazole and sulfacetamide. Doxorubicin did not provide a suitable positive control for apoptosis (not shown). However, the use of other experimental methods including DNA laddering test (explained below) and fluorescent microscopy (mentioned above) supported the flow cytometric data indicating of the absence of apoptosis in drug-treated cells.

Sulfathiazole and sulfacetamide do not cause DNA fragmentation

T-47D cells were exposed to LC₅₀ concentration of sulfathiazole and sulfacetamide for 48h and cellular DNA was analyzed by gel electrophoresis for DNA inter-nucleosomal fragmentation, i.e. DNA laddering, as an indication of apoptosis (Alberts *et al.*, 2008). Figure 4 shows that despite of the existence of DNA laddering for camptothecin-treated cells (positive control) similar patterns were not present in sulfathiazole and sulfacetamide treated cells. Lack of DNA fragmentation is consistent with the flow cytometric and fluorescent microscopic data, confirming the absence of apoptosis in treated T-47D cells.

Caspase-3 expression and activation assays in T-47D breast cancer cells incubated with sulfathiazole and sulfacetamide

Following of the absence of apoptosis in drug-treated T-47D cells, caspase-3 expression and activation was checked. Caspase-3 is a key enzyme involved in most apoptotic pathways via activation or inactivation of a range of cellular molecules that are involved in type I programmed cell death - apoptosis (Alberts *et al.*, 2008). Comparison between the average fluorescence intensities for the samples compared to control showed a 2.2-fold increase in the enzyme activity of caspase-3 in the presence of sulfathiazole (Figure 5). Furthermore, real time data indicated an increase in caspase-3 expression in sulfathiazole treated cells, which was absent in cells incubated with sulfacetamide (Figure 7 and Table 2; latter available in Online Resource 6). Absence of any increase in caspase-3 *expression* in sulfacetamide treated cells was consistent with the absence of a significant increase in caspase-3 *activity* (~1.4 fold increase) (Figures 7B and 5).

Cell cycle analysis in the sulfacetamide and sulfathiazole treated cells

No or few cells appearing in the G₀/sub-G₁ region confirmed that sulfacetamide and sulfathiazole treatment did not induce apoptosis in inhibiting T-47D cell survival (not shown). Moreover, no significant change in dissipation of the cell populations in different phases of the cell cycle (G₁, S and G₂), relative to the control emphasized that a 50% in viability after 48 h incubation could not have been caused by cycle arrest (Figures 6A and 6B). Doxorubicin as a positive control showed detectable accumulation of S phase cells (middle bell-shaped curve in Figure 6A).

Expression level of pro- and anti- apoptotic genes in the presence of sulfathiazole and sulfacetamide

Figure 7 shows that the expression levels of some pro-apoptotic and anti-apoptotic genes such as AIF, bcl-2, DFF40 and DFF45, determined by real time RT-PCR, were altered in

cells incubated with sulfathiazole and sulfacetamide. These transcriptional changes have significant impact on apoptosis and are discussed later.

Autophagy is induced by sulfathiazole and sulfacetamide in T-47D cells

Figure 7 shows that ATG5 expression level increased in the cells incubated with sulfathiazole and sulfacetamide. ATG5, in combination with ATG12, is involved in the biogenesis of autophagic vesicles (Roy and Debnath, 2010). A rigorous increase in ATG5 expression in cells incubated with sulfathiazole and sulfacetamide suggests an increase in autophagosome formation in the autophagy pathway. Moreover, the increased expression of p53 and DRAM indicates that the autophagy induction was via this pathway.

Discussion

We have demonstrated that sulfathiazole and sulfacetamide are suitable suppressors of human breast cancer T-47D cell proliferation by significantly reducing cell viability. Increased expression of the anti-apoptotic bcl-2 gene without alteration in AIF expression level in sulfathiazole and sulfacetamide treated cells occurred (Figure 7 and Table 2; available in Online Resource 6). There was an absence of apoptosis in T-47D cells under our treatment conditions. This was supported by the low number of apoptotic cells, the preferred distribution of treated cells in Q₃ region of flow charts, the lack of DNA fragmentation, and no alteration in PARP1 expression (Figures 2, 3, 4 and 7). Several studies have broadened the role of poly-ADP-ribosylation in cell killing, showing that PARP1 activation occurs during AIF induced apoptosis (Yu *et al.*, 2002). Thus, the constant expression of PARP1, along with a similar expression of AIF in drug treated cells, also supports the lack of apoptosis in drug-treated T-47D cells.

Increase in caspase-3 activity in sulfathiazole treated cells was not followed by induction of apoptosis. This could be explained by upregulation of the DFF45/DFF40 expression ratio (~1.6), which can block caspase-3 effect on DNA fragmentation. DFF40 (CAD) is the natural DNase activated in apoptosis via caspase-3-mediated degradation of DFF45 (iCAD, natural inhibitor of CAD). Thus, DFF40, which is trapped in drug treated cells by the increased protein level of DFF45, cannot exert its hydrolytic action, or induce DNA fragmentation and apoptosis (Liu *et al.*, 1997). Moreover, increased DNA-PK (a double-strand break repair enzyme) expression in sulfathiazole and sulfacetamide treated cells further counteracts DNA fragmentation and induction of apoptosis (Figure 7 and Table 2; available in Online Resource 6).

Flow cytometric graphs after DAPI staining indicate no significant changes in cell cycle distribution of sulfathiazole and sulfacetamide treated cells compared to controls (Figure 6). Therefore, cell cycle arrest is not a mechanism for anti-proliferative action of sulfacetamide and sulfathiazole on T-47D cells.

The expression of ATG5, an essential protein in early stage of autophagosome formation, was enhanced by sulfathiazole and sulfacetamide treatment, indicating that autophagy was increased in T-47D cells. Autophagy is a 2-phase process that exerts both tumor suppressive and prosurvival effects (Roy and Debnath, 2010). The tumor suppressive effect of

autophagy manifests through type II programmed cell death also called death inducing autophagy. In this type of autophagy, Akt/mTOR pathway is inhibited by several factors, including chemopreventive drugs (Salazar *et al.*, 2009). Prosurvival effects of autophagy guarantees viability of the cancer cells via increased tolerance against environmental stresses, including starvation and chemotherapeutic drugs (Roy and Debnath, 2010). Inhibition of AKT/mTOR pathway and its increased activity was respectively ascertained in our sulfathiazole and sulfacetamide treatments, as discussed below.

p53 promotes transcription of negative regulators of the mTOR pathway, including AMPK β , TSC2 and PTEN, and has a critical role in induction of autophagy (Roy and Debnath, 2010). During starvation, activation of AMPK inhibits Akt/mTOR pathway and induces autophagy (Shang and Wang, 2011). p53 can also promote autophagy in an mTOR-independent manner via the transcriptional upregulation of its downstream target, DRAM. This is called mTOR-independent p53/DRAM pathway (Roy and Debnath, 2010). The presence of sulfathiazole and sulfacetamide results in increased p53 and DRAM expression levels (Figure 7 and Table 2, available in Online Resource 6). Thus, induction of autophagy in our experiments might occur via p53/DRAM pathway. In the presence of sulfathiazole, induction of autophagy occurred in parallel with downregulation of AKT1, AKT2 and mTOR, and upregulation of PTEN. Thus, blockade of Akt/mTOR pathway and exertion of death inducing effects occurred through p53/DRAM in an Akt/mTOR-dependent manner. Similarly in our study, autophagic cell death in the presence of another sulfonamide family member, sulfabenzamide, has been reported (Mohammadpour *et al.*, 2012). Triggering of p53/DRAM pathway not only induced death autophagy in the presence of sulfathiazole in an Akt/mTOR dependent manner, but could induce cytoprotective autophagy in the presence of sulfacetamide. In the presence of sulfacetamide, lower expression level of p53 and DRAM compared to the sulfathiazole treatment occurred in parallel with upregulation of Akt/mTOR pathway confirming cellular behavior to sustain viability via autophagy (i.e. cytoprotective autophagy). Figure7 shows that the amount of p53 and DRAM expression in the presence of sulfathiazole was significantly greater than that of sulfacetamide. Thus, greater expression of p53 and DRAM genes could trigger death inducing autophagy via downregulation of Akt/mTOR pathway in the sulfathiazole treatment. However, in the presence of sulfacetamide, lower expression of p53 and DRAM genes occurs along with positive regulation of Akt/mTOR pathway, resulting in engagement of cytoprotective autophagy. Thus, transition between the 2 types of autophagy in the cells may be regulated by the level of p53 and DRAM genes.

T-47D cells contain only a single copy of the p53 missense mutation at residue 194 within the zinc-binding domain (Schafer *et al.*, 2000). The mutant p53 may not only lose its natural anti-tumor activity, but also acquire additional pro-oncogenic gain-of-function activities via binding to the wild type p53 and inhibiting its normal function (Lim *et al.*, 2009). Interestingly, in the presence of sulfathiazole and sulfacetamide, the anti-tumor and pro-oncogenic gain-of-function activities of mutant form of p53 should return to its normal condition (i.e. wild type p53) to induce autophagy. This is very similar to the mechanism of action for some anti-tumor drugs reactivating mutant p53 to kill cancer cells (Lambert *et al.*, 2009).

Death inducing effects of autophagy after inhibition of Akt/mTOR pathway has been attributed to the enhanced apoptosis (Salazar *et al.*, 2009). We found autophagy induction in the sulfathiazole and sulfacetamide treatments was not accompanied by apoptosis. Autophagy may protect against apoptosis by eliminating damaged mitochondria that release pro-apoptotic signaling molecules, e.g. cytochrome c, Apaf, and reactive oxygen species (Lockshin and Zakeri, 2004). Alternatively, some believe that when apoptosis is blocked, autophagy is triggered and vice versa (Chen and Karnatza-Wadsworth *et al.*, 2009). These possibilities are consistent with our findings regarding lack of apoptosis in drug-treated cells and activation of autophagy. Furthermore, induction of autophagy by PUMA (the p53-inducible BH3-only protein) depends on Bax/Bak and can be reproduced by overexpression of Bax (Yee *et al.*, 2009). These authors found Bax overexpression and autophagy activation, which also occurred in our studies via increased expression of Bax in sulfathiazole treated cells (Figure 7 and Table 2; available in Online Resource 6).

Induced autophagy after sulfathiazole and sulfacetamide treatment occurred without any distinctive arrest in a phase of the cell cycle (Figure 6). In a chemopreventive drug stress, cytoprotective autophagy degrades mitochondria promoting cell fitness via decreasing metabolism, resulting in a decreased rate of proliferation without arrest in any distinct phase of the cycle. This means that the rate of passage through all phases of the cell cycle seems to decrease in the same proportion. Reduced proliferation can provide an opportunity for the cells to counter the possible toxic effects of a drug in dividing cells. Thus, mechanism of action for sulfacetamide must be imposed by such a mechanism that decreases the rate of cell proliferation.

The Death-Associated Protein Kinase (DAPK) family may form multi-protein complexes capable of transmitting apoptotic or autophagic cell death signals in response to various cellular stresses (Gozuacik and Kimchi, 2006). Our real time RT-PCR data showed that DAPK gene was completely silent in untreated as well as sulfathiazole and sulfacetamide treated cells. DAPK expression is silenced in several human malignancies and can stimulate autophagy by multiple mechanisms following its activation (Maiuri *et al.*, 2010). Thus, sulfathiazole and sulfacetamide triggers autophagy in T-47D cells via a DAPK independent pathway. Furthermore, BECN1 expression increases, in addition to an increase in Bcl-2 expression and caspase-3 activity (Figures 5 and 7). Bcl-2 can inhibit BECN1 activity for autophagy induction (Kang *et al.*, 2011). Cleavage of BECN1 by caspase-3 also prevents pro-autophagic activity of BECN1 (Kang *et al.*, 2011). Thus, autophagy induction in T-47D cells in the presence of sulfathiazole and sulfacetamide occurs in a BECN1-independent manner.

Other supplementary methods such as Western blotting could provide further documentation supporting the real time RT-PCR data. However, we found that the changes in RNA transcripts were in good agreement with the expected cell behavior. Thus, suggesting that the protein expression levels or their activities should change in parallel with the RNA levels in the cells. Therefore, although there are some exceptions, evaluating RNA transcripts along with biological data provides sufficient evidence to support changes in protein expression levels in these cells.

Conclusions

We have demonstrated that sulfathiazole and sulfacetamide do not induce apoptosis or cell cycle arrest in T-47D breast cancer cells. However, autophagy is a good candidate in the 50% reduction in drug treated cell viability compared to control cells. Autophagy induction was induced in 2 different forms based on the expression level of p53 and/or DRAM genes. These 2 forms of autophagy include death inducing in sulfathiazole and cytoprotective in sulfacetamide treated cells. There have been many attempts to distinguish the molecular pathways leading to death inducing or cytoprotective autophagy. Our data suggest that expression level of p53 and/or DRAM genes may control some switching between the 2 types of autophagy.

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Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog
mTOR	mamalian Target of Rapamycin
DAPK	Death-Associated Protein Kinase
DRAM	Damage Regulated Autophagy Modulator
ATG5	Autophagy related gene 5
BCLN1	Bcl-2 Interacting protein 1
PARP1	Poly ADP-Ribose Polymerase 1
DFF40/CAD	DNA Fragmentation Factor 40/ Caspase-Activated DNase
Bax	Bcl2-Associated X protein
Bcl-2	B-Cell Lymphoma 2
AIF	Apoptosis Inducing Factor
DFF45/iCAD	DNA Fragmentation Factor 45/inhibitor of Caspase-Activated DNase
Cdc2	Cell Division Cycle protein 2
ARF	ADP-ribosylation Factor
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
AMPKβ	AMP-activated Kinase beta
TSC2	Tuberous Sclerosis Complex 2
PTEN	Phosphatase and Tensin homolog
Bif-1	Bax interacting factor 1

DNA-PK	DNA-dependent Protein Kinase
RPMI	Roswell Park Memorial Institute
G₁, G₂, M and S	phases of the cell cycle
SDH	Succinate <u>d</u> ehydrogenase

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A

B

Figure 1. Viability plot of T-47D cells drawn based on increased concentration of sodium sulfacetamide (A) and sodium sulfathiazole (B)

Percent viability of drug treated cells calculated relative to the control and reported at different concentration range of sodium sulfacetamide and sodium sulfathiazole (0–50 mM) at each incubation time, shown at the upper right of the figure. Each point in the figure relates to the mean value of 3 independent experiments. Above each curve the related correlation coefficient (r^2) is brought until the goodness of fit for the selected mathematical function used to interpolate the experimental points.

Insets: chemical structure of sulfacetamide (A) and sulfathiazole (B).

A

B

Figure 3.

A- 2-dimensional plot of Annexin V-FITC against PI related to the flow cytometric experiments. Analysis border was ascertained using FSC/SSC plot related to the untreated cells. Determination of the borders of the 4 quadrants (Q₁–Q₄) was performed based on the placing of the maximum dots in the Q₃ region of control sample. Apoptosis and necrosis were not significantly induced by sodium sulfacetamide and sodium sulfathiazole. **B- Histogram of the percent values of the cells against flow cytometric results of Annexin V-FITC and PI double staining.** The last triplet column at the right side of the histogram corresponds to Q₃ region (live cells) and scaled at the right y axis. Other triplet columns in the histogram correspond respectively (from left to right) to the Q₁ (necrotic cells), Q₂ (late apoptotic cells), Q₄ (early apoptotic cells), Q₁+Q₂ (in some reports it shows percent value of necrotic cells) and Q₂+Q₄ (total percent value of apoptotic cells). Each set of triplet columns from left to right relates to the control, sulfacetamide and sulfathiazole treated samples.

Figure 4. Demonstration of DNA fragmentation in T-47D cells exposed to sodium sulfacetamide and sodium sulfathiazole by agarose gel electrophoresis

The lanes from left to right relate to positive control (camptothecin treated DNA extract), sulfacetamide, sulfathiazole, negative control and size marker. Lacking of laddering pattern in sulfathiazole and sulfacetamide treated samples beside the negative control indicated lacking of apoptosis.

Figure 5. Caspase-3 activity assay

Enzyme activity in the control, sodium sulfathiazole, and sodium sulfacetamide treated cells were 2.18 ± 0.13 , 4.76 ± 0.43 and 3.55 ± 0.3 nM/h, respectively.

A

B

Figure 6.

A- Effects of sodium sulfacetamide and sodium sulfathiazole on cell cycle distribution.

FL4-A indicates the area under the registered electrical signal of each stained cell when it passes through the laser beam. The bell-shaped curves from left to right relate to G₁, S, G₂/M phases of the cell cycle in control, doxorubicin, sulfacetamide and sulfathiazole treated cells.

B- Histogram of the percent values of the cells in each phase of the cell cycle.

Percent values of the cells in the G₁, S and G₂/M phases of the cell cycle are shown.

Each set of triplet columns from left to right relates to the control, sulfacetamide and sulfathiazole treated samples. Doxorubicin was utilized here as positive control causing main transition from G₁ to S (24%) and subsidiary lower transition from G₁ to G₂/M (13%).

The related columns of the phases were not shown in the figure for simplification.

A

B

Figure 7. Quantitative real time RT-PCR analysis histograms

Real time RT-PCR of the selected genes for sulfacetamide (A) sulfathiazole (B) treated cells was determined as described in the Methods. The relative amount of target material was quantified compared to the reference genes using the comparative C_T ($-C_T$) method. The Statistical Significant differences are indicated with * and ** for $p < 0.05$ and $p < 0.01$, respectively.