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A novel bis-aryl urea compound inhibits tumor proliferation via cathepsin D-associated apoptosis

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Derivatives of bis-aryl urea have been widely investigated for their various biological activities, such as antiviral, anti-inflammatory and antiproliferative. We evaluated a new chemical entity consisting of bis-aryl urea moiety, N69B, for its anticancer activities and explored their underlying molecular mechanism. The compound inhibited proliferation of multiple types of murine and human cancer cells *in vitro*, and reduced tumor growth in mouse 4T1 breast tumor model *in vivo*. Protein microarray analysis revealed and western blot confirmed that the compound significantly increased protein levels of cathepsins, especially cathepsin D, a lysosomal aspartyl protease known to have various pathophysiological functions. Further studies showed that the compound induced tumor cell apoptosis through the Bid/Bax/Cytochrome C/caspase 9/caspase 3 pathway, in which cathepsin D appeared to be a main mediator. Unlike kinase inhibition commonly seen with many other anticancer bis-aryl urea derivatives, this unique mechanism of N69B may suggest potential of the compound as a novel anticancer drug.

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Introduction

With the introduction of the first bis-aryl urea drug sorafenib for the treatment of liver and kidney tumors, bis-aryl ureas have become a new hotspot in anticancer drug research [1]. Compounds with an aryl urea scaffold can have highly diverse biological activities, including antiatherosclerotic [2] and antibiotic [3] effects in addition to anticancer activities [4]. As an anticancer drug with multiple targets, Sorafenib can block the RAF/MEK/ERK pathway and inhibit VEGFR to restrain the proliferation of tumor cells [1]. Various other compounds containing bis-aryl urea groups have been successively synthesized [5] and have shown anticancer activities mainly through kinase inhibition. These include tyrosine kinase inhibitors ABT-869 and KRN951 [6–8], which inhibit VEGFR and PDGFR, and serine-threonine kinase-targeting small molecules, which selectively inhibit Lim kinas [9]. The compound N69B is a new chemical entity, in which the bis-aryl urea group is coupled with a trisubstituted s-triazine. We wanted to evaluate its anticancer potential on various types of cancer cells and to understand the underlying molecular mechanism.

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Materials and methods

Drug preparation

Chemical name of the compound N69B is (E)-1-(4-(4-Methylpiperazine-1-carbonyl) phenyl)-3-(4-((4-morpholino-6-styryl-1, 3, 5-triazin-2-yl)amino)phenyl)urea.The compound was synthesized at Bellen Chemistry Co, Ltd. and provided by Luoda Biosciences, Inc. The compound structure and purity analyses are shown in Supplementary Fig. 1, Supplemental digital content 1, *http://links.lww.com/ACD/A327*. It has the formula of $C_{34}H_{37}N_9O_3$, the measured molecular weight of 619.8 (MS, $[M+H]^+$) and the purity of 97.5% (HPLC).

The compound was either dissolved in DMSO (Solarbio, Beijing, China) or suspended in 0.5% CMC-Na (Solarbio, Beijing, China) for in-vitro and in-vivo studies. The stock solution of 20 mM was stored at –20°C and diluted with RPMI media (Gibco, USA) to working concentrations prior to use; the stock suspension of 100 mg/ml was stored at 4°C for a maximal duration of one week and warmed at room temperature prior to use.

Cell culture

Human non-small cell lung cancer cell lines A549 and H522, human gastric cancer cell lines AGS and MKN45, human hepatoma cancer cell line SMMC-7721, human glioma cell line U87MG, murine mammary cancer cell line 4T1 and murine colon cancer cell line CT26WT were purchased from Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). They were maintained at

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37°C in RPMI1640 medium containing 10% fetal bovine serum (FBS, Sigma, USA), 100 U penicillin and 100 mg/ml streptomycin (Gibco) in a humidified atmosphere of 5% CO2.

Cell proliferation assay

The viability of cells was determined by Cell Counting Kit, CCK-8 (Dojindo, Japan). Briefly, cells in the exponential growth phase were harvested; suspension of 5×10^3 single cells were seeded in each well of 96-well plates and incubated at 37°C overnight. The cultures were added respectively with DMSO as vehicle control and the compound N69B of different concentrations, and grown for 24, 48 or 72 h. The cells were added with CCK-8 and incubated with for 1–2 h.

The relative number of viable cells was determined by measuring the absorbance with Multi-function microplate reader (PerkinElmer, EnSpire, America) at a wavelength of 450 nm. Triplicates were run in three independent tests.

Colony-forming assay

Cells were seeded in six-well plates at 1×10^3 cells per well with different concentrations of N69B, and incubated at 37°C for 10 days. The cells were stained with 2% crystal violet for 15 min and then washed twice with PBS. Plates were dried at room temperature, and cell colonies formed in each plate were counted. The assay was carried out in triplicates.

Animals

Eighteen female BALB/c mice (18–20 g) aged 6–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in the Laboratory Animal Center of Nanjing University of Chinese Medicine. Animals were maintained at 22 ± 2°C on a regular light–dark cycle with free access to food and water. All animal experiments were approved by the Nanjing University of Chinese Medicine ethics committee. Animal care was provided in accordance with guidelines approved by the IACUC.

In-vivo tumor growth

A total of 2×10^5 4T1 cells were injected subcutaneously into the left mammary gland of each mouse. The mice were then randomly divided into three groups, six mice per group. Starting from the second day after inoculation, mice in the intraperitoneal injection group and intragastric administration group were given 100 mg/kg of N69B daily. Control mice were given the same volume of solvent every day. The longest length (L) and the widest orthogonal width (W) of each tumor were measured three times a week, and tumor volumes (mm^3) were calculated by the formula $L \times W \times W/2$. Twenty-one days after the inoculation, the mice were euthanized. The tumors were dissected out, photographed and weighed.

Apoptosis assay

Cell apoptosis was determined by the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) according to the manufacturer's instruction. Briefly, cells were seeded in six-well plates at 3×10^5 cells per well, incubated at 37°C overnight, then added with the compound N69B of different concentrations, and continued in culture for 24 h. Cells were harvested, washed with cold PBS and counted. Samples of 1×10^6 cells were suspended with 100 µl Binding Buffer, then added with 5 µl FITC Annexin V and 5 µl PI and incubated in the dark for 5 min at room temperature. Each sample was added with 400 µl Binding buffer and analyzed by flow cytometry (Accuri C6; BD Biosciences).

Western blot

Cells were cultured with different concentrations of N69B for 48 h, then collected and lysed by RIPA buffer containing PMSF (Beyotime Biotechnology, Shanghai, China). Lysates were cleared by centrifugation to remove insolubles; protein concentrations were determined by the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology). Forty micrograms of proteins were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane (Merck Millipore). The membrane was blocked by 5% milk for 2 h, and then incubated with primary antibody at 4°C overnight. The membrane was washed three times, then incubated with secondary antibody at room temperature for 1 h. The bands were detected by Gel Imaging System (Bio-Rad, USA), and measured by ImageJ. The primary antibodies used were rabbit anti-β-actin, EGFR, bFGF, cathepsin D (CTSD), Bid, cytochrome C (Cyt C), caspase 3, caspase 8 (Cell Signaling Technology, USA), Bax, caspase 9, cleaved caspase 9 (Affinity Biosciences, USA)

Protein chip analysis

Lysates from A549 cells treated with 1.5 µM of N69B or equal volume of DMSO were extracted with Lysis Buffer 17(R&D Systems, Abingdon, UK) supplemented with 10 µg/ml of Aprotinin (Sigma, Shanghai, China), 10 µg/ml of Leupeptin (Sigma, Shanghai, China), and 10 µg/ml of Pepstatin (Sigma, Shanghai, China). A total of 200 µg of the lysate was run on each array of the Human XL Oncology Array Kit (R&D Systems). The procedures were followed strictly in accordance with instructions from the manufacturer. The membrane chips were exposed to x-ray films for 8 min. Pixel densities on the developed x-ray film (hu.q, HQ-320XT, China) were quantified by a transmission-mode scanner (EPSON, Beijing, China) and analyzed by Image J software.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 5.0. Data were presented as mean ± SD. Differences between groups were analyzed by Student's *t* test. $P < 0.05$ was considered statistically significant.

Result

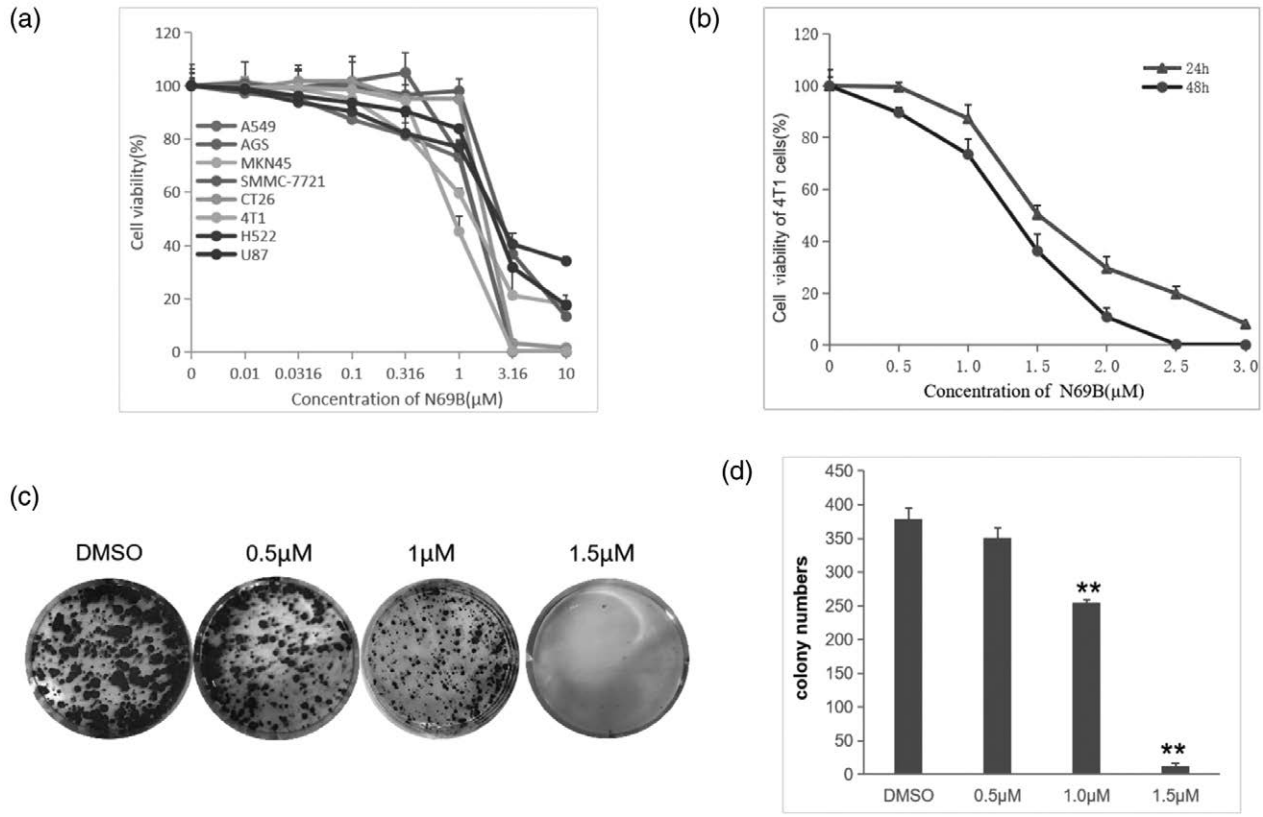
The compound N69B inhibits cell proliferation of various cancer types *in vitro*

The compound N69B is a new chemical entity consisting of the bis-aryl urea scaffold linked to a substituted s-trazine (Supplementary Fig. 1, Supplemental digital content 1, *http://links.lww.com/ACD/A327*). To test its anticancer potential, we selected cell lines derived from human cancers of lung, stomach, liver and brain, and mouse cancers of breast and colon. The compound strongly inhibited the proliferation of all the tested cancer cells in a dose-dependent manner (Fig. 1a). Sensitivities to the compound varied among different cancer lines, indicating that the growth inhibition was not due to nonspecific killing. The dose responsive curves were relatively steep, and IC_{50}s were in a narrow range between 1 and 3 μM for all cancer types, suggesting that the drug target may have multiple sites for binding or have concentrations much higher than the dissociation constant (Kd) of the binding compound, or the compound may undergo a physical phase transition as its concentration is raised [10]. We took the most sensitive line, mouse 4T1 murine cancer cell for further testing. The longer the cells were treated with the compound, the stronger inhibitory effects were achieved (Fig. 1b). The clonogenicity assay showed that N69B at 0.5 μM, only slightly reduced the number of colonies, but at 1 μM, significantly inhibited colony formation and, at 1.5 μM, allowed virtually no colony to form (Fig. 1c and d), consistent with the steep dose–responsive curve shown in the proliferation assay.

N69B inhibited tumor growth *in vivo*

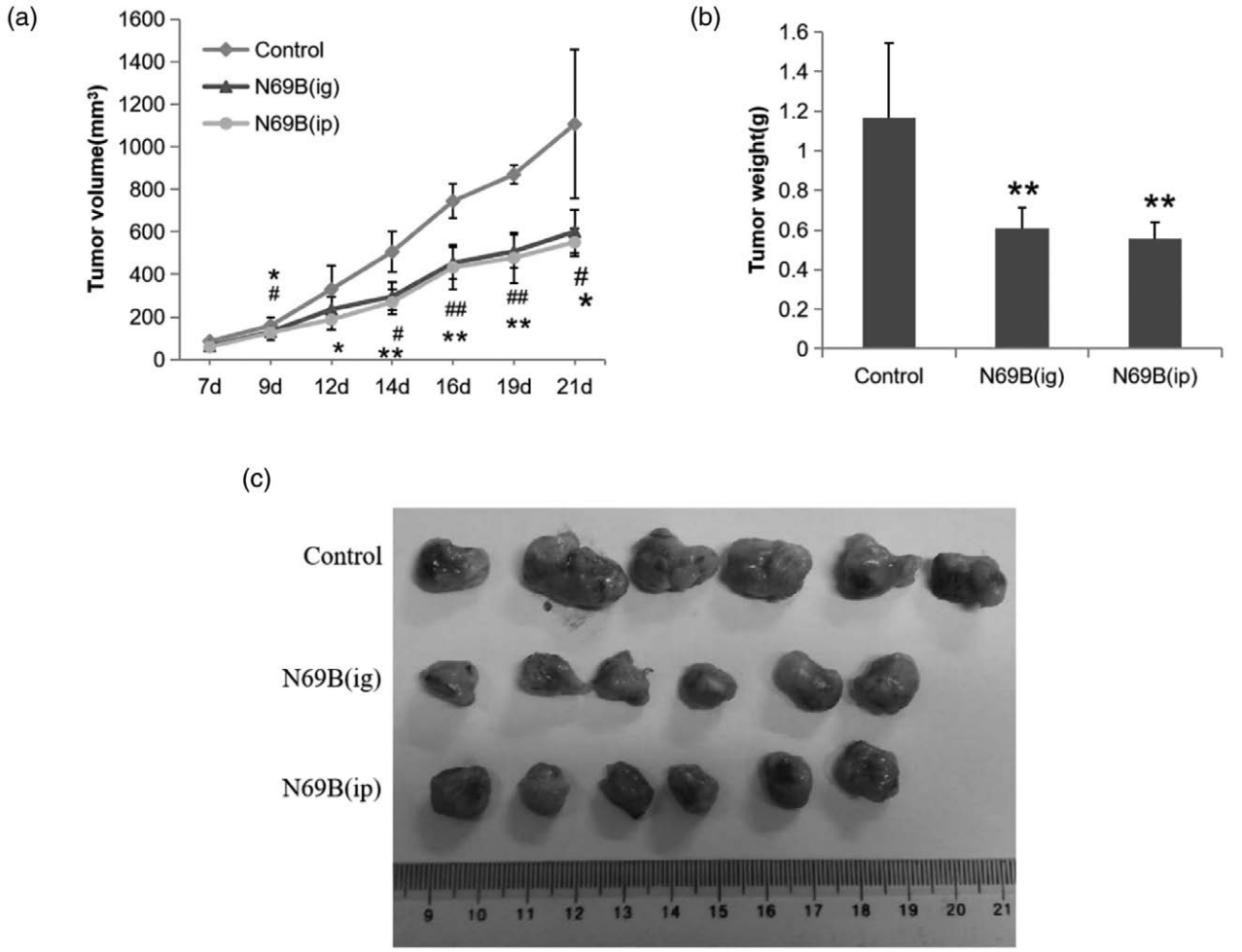
To evaluate the druggability of the compound N69B, we selected murine mammary cancer 4T1 cells for in-vivo testing, as no cancer-type specificity was observed *in vitro*. The cells were inoculated subcutaneously into the mammary gland of female BALB/c mice. The compound was administered by gavage and by intraperitoneal injection, respectively. Both of the delivery routes resulted in significant delay in cancer growth and reduction in tumor volumes (Fig. 2a). The study was terminated 3 weeks after cancer cell inoculation. The tumor weights were found markedly reduced in both N69B treatment groups (Fig. 2b and c). Comparable effects of the oral and the intraperitoneal drug delivery routes may suggest that the compound has good oral

Fig. 1



The compound N69B inhibited cancer cell growth and clonal forming ability. (a) Dose–responsive inhibition on cell proliferation of various cancer types. (b) Exposure time-dependent inhibition on proliferation of 4T1 cells. (c) and (d) Inhibition on clone formation of 4T1 cells. The data are expressed as mean ± SD of three independent experiments. Significance: $^{**}P < 0.01$ vs. negative control.

Fig. 2



N69B inhibit tumor growth of mammary carcinoma 4T1 cells *in vivo*. (a) Tumor volumes over the course of the study. (b) and (c) Tumor mass dissected and weighted at day 21. Tumor volumes and weights were significantly smaller in each N69B group compared with control group. Data are presented as mean ± SD, $n = 6$. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ i.p, vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ i.g. vs. control group.

bioavailability and can be readily absorbed through gastrointestinal tract.

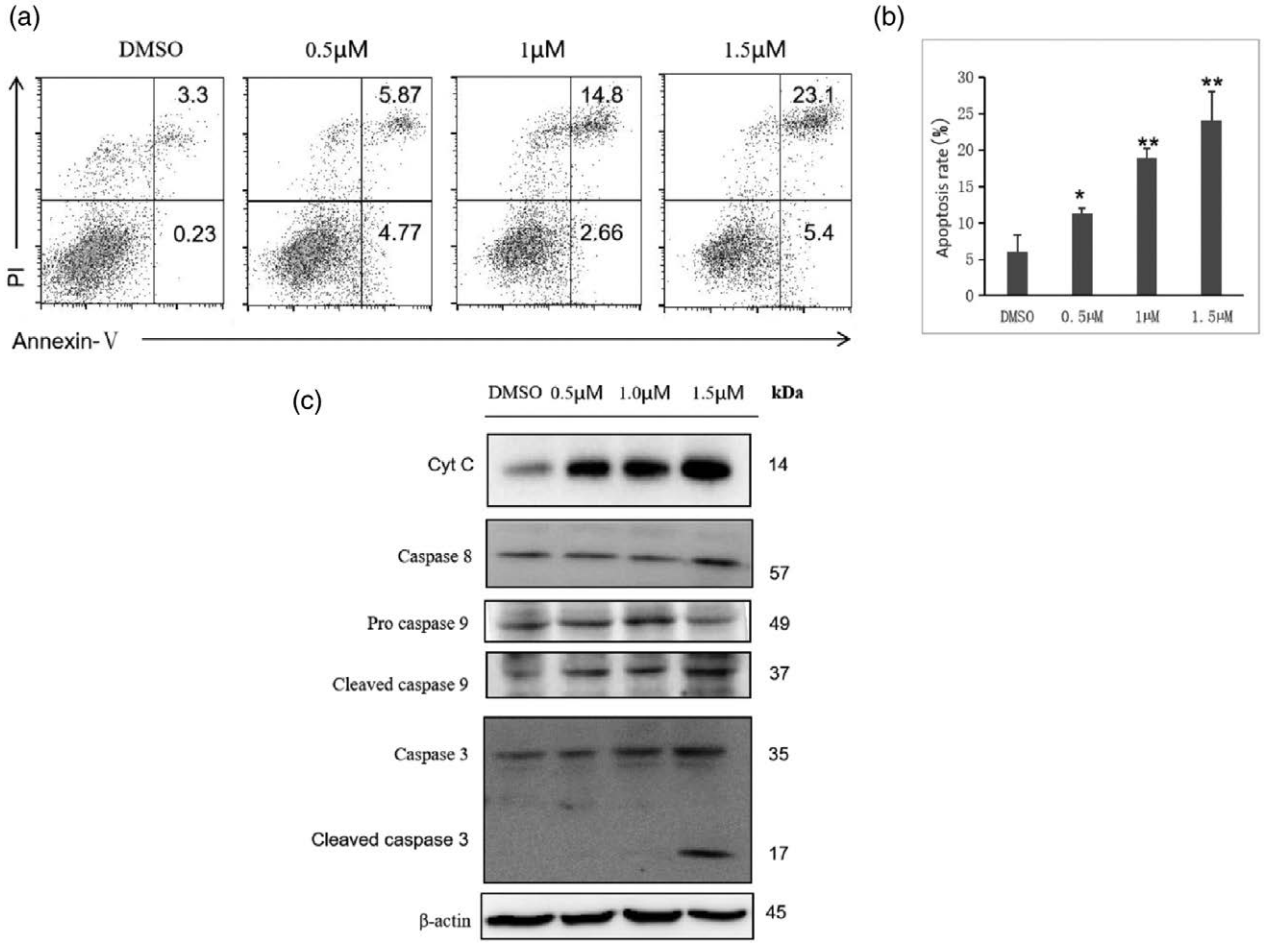
N69B induced cancer cell apoptosis

The broad inhibition on various types of cancer cells suggests that the compound N69B may act through a mechanism involving conserved pathways. Apoptosis is a common pathway leading to cell death. We assessed the cell apoptosis by Annexin V/PI double staining of 4T1 cells. The treatment with N69B for 48 h resulted in a dose-dependent increase in the number of Annexin V-positive 4T1 cells (Fig. 3a and b). During apoptosis, cytochrome c (Cyt C) release often occurs, which in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death [11]. Indeed, N69B treatment led to concentration-dependent increase in Cyt C (Fig. 3c). The downstream proteins, cleaved caspase 9 and cleaved caspase 3 also increased in a dose-dependent manner. Caspase 8 levels showed no significantly change at lower doses, but elevated in response to the high dose N69B treatment (Fig. 3c).

Cathepsin D is associated N69B-induced apoptosis

In order to identify mediators of the apoptosis induced by N69B, we profiled major cancer-related proteins in human lung cancer A549 cells using the Human XL Oncology Array (RnD Systems, USA). N69B-treated cells showed no changes in oncoproteins that sorafenib or other bis-aryl urea commonly targets, such as EGFR/FGFR signaling pathways. Instead, N69B showed effects on only a few proteins, including a decrease in survivin, a member of the inhibitor of apoptosis family that inhibits caspase activation; an increase in FoxO1, a transcription

Fig. 3



N69B inhibited cell proliferation of 4T1cellls via the induction of apoptosis. (a) FACS analysis of apoptotic cells by PI and Annexin V double staining. (b) Quantitative measurement of the proportion of early and late stage apoptotic cells. Data are presented as means ± SD from three independent experiments. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. negative control. (c) Western blot of apoptosis-related proteins.

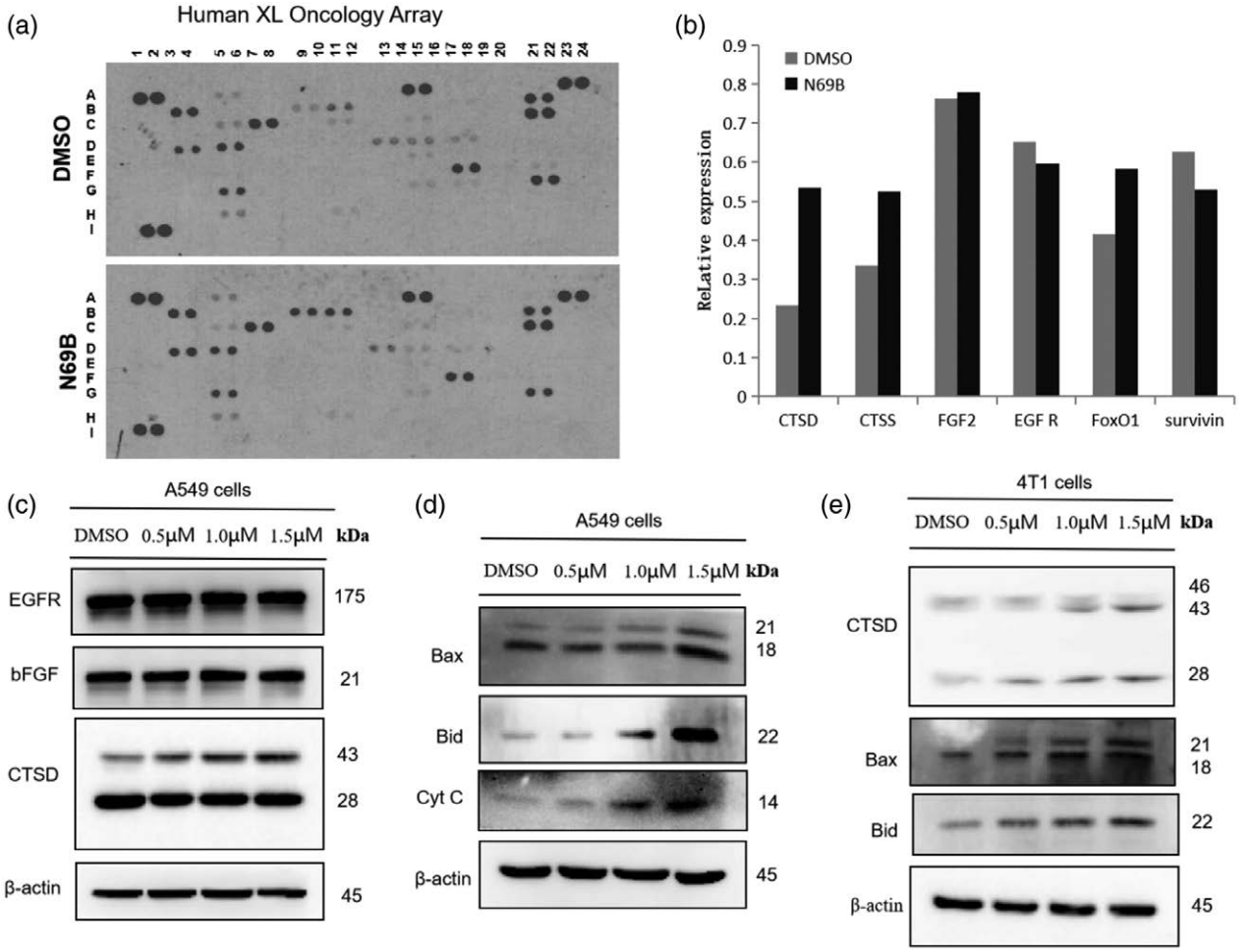
factor that is the main target of insulin signaling and regulates metabolic homeostasis in response to oxidative stress; and increases in Cathepsin S (CTSS) and CTSD, a group of enzymes that have a key role in cellular protein turnover. Among them, CTSD had the most significant alteration (Fig. 4a and b, Supplementary Fig. 2 and Table 1, Supplemental digital content 1, *http://links.lww.com/ACD/A327* and).

Further study by Western blot confirmed the finding that CTSD, especially the form of pro-cathepsin, was upregulated, not only in N69B-treated human lung cancer A549 cells, but also in N69B-treated murine mammary cancer 4T1 cells. Similar to that in human A549 cells, proapoptotic proteins Bax and Bid were also increased in murine 4T1 cells (Fig. 4c–e), suggesting there may be a connection between CTSD and cell apoptosis induced by N69B, regardless of the species and cell types from which the cancer originates.

Discussion

As a novel bis-aryl urea compound, N69B can inhibit the proliferation of various cancer cells both *in vitro* and *in vivo*, similar to other known bis-aryl urea derivatives. The underlying molecular mechanism, however, appears to be very different. Sorafenib, a well known bis-aryl urea, has been widely used for the treatment of liver, kidney and thyroid cancers, and in combination with other drugs for the treatment of various types of cancer. It induces cancer cell apoptosis through inhibiting multiple kinases in angiogenic pathway and in cell proliferation, with activity against RAS/RAF kinases and several receptor tyrosine kinases, including VEGFR, PDGFR, FLT3, Ret and c-Kit. However, the potent anticancer activity of Sorafenib also associates with its mechanism-based toxicities that can severely impact the physical, psychological and social well-being of patients [12]. Efforts have been devoted to finding more potent and less toxic compounds, still, most of those bis-aryl ureas currently

Fig. 4



CTSD is a mediator in N69B induced apoptosis. (a) Semiquantification of oncoproteins in N69B-treated cells by the Human XL Oncology. The lysates were obtained from A549 cells treated for 48h with either DMSO or 1.5 μM of N69B. Coordinates B9, B10: CTSD; B11, B12: CTSS; C21, C22: FGF; B21, B22: EGFR; D3, D4: FOXO1; G21, G22: Survivin. See Supplementary Fig. 2 and Table 1, Supplemental digital content 1, *http://links.lww.com/ACD/A327* and for details. (b) The graph summarizes the relative signal intensity of indicated proteins, among which CTSD varied most significantly. (c) and (d) Western blot for selected proteins from human A549 cells, EGFR, bFGF, CTSD, Bax, Bid and Cyt C. (e) Similar alteration of CTSD, Bax and Bid protein levels in murine 4T1 cells treated with N69B. CTSD, cathepsin D; CTSS, cathepsin S.

under development are also kinase inhibitors and are not much distinguished from Sorafenib. While the compound N69B inhibits cancer cell proliferation via the induction of caspase-dependent apoptosis, it appears to be through a mechanism other than kinase inhibition. In order to elucidate molecular mechanisms, we used protein chip analysis to identify pathways involved in N69B-induced apoptosis. A protein microarray was applied consisting of 84 human cancer-related proteins, such as proliferation-associated proteins, migration-associated proteins, apoptosis-associated proteins and inflammation-associated proteins. As the result showed, VEGF, the main component of angiogenic pathway and a target of sorafenib [6], did not change in the cells exposed to N69B at effective concentration. EGFR, a key receptor tyrosine kinase promoting cell proliferation and opposing apoptosis, which is a target of Sorafenib and the target of other successful anticancer medicine, such as erlotinib and gefitinib [13, 14], also did not change in cells treated by N69B. Instead, cathepsins, especially CTSD, were significantly elevated after N69B treatment.

CTSD is the only aspartyl protease ubiquitously expressed in all human cells. The mature protein is distributed in lysosomes where it selectively and partially degrades specific proteins and activates precursors of proteins that are essential to proper cellular functions [15]. Originally considered as a housekeeping enzyme, CTSD has now emerged as a multifunctional protein, involved in myriad physical and pathological processes.

Earlier studies suggested that CTSD could induce apoptosis in presence of cytotoxic factors [16–20]. The two

proapoptotic Bcl-2 family proteins Bid and Bax are substrates of CTSD during apoptosis [21]. Upon activation, Bid is truncated by proteolytic cleavage to form tBid and then translocated to mitochondria where tBid binds to its mitochondrial partner Bak to release cytochrome C [22]. CTSD can also directly activate Bax and cytochrome C to induce the intrinsic pathway of apoptosis [22]. Our study showed that, in the cancer cells undergone apoptosis after N69B treatment, elevated CTSD was correlated with increased levels of Bid, Bax and cytochrome C (Fig. 4c and d), suggesting that N69B may induce apoptosis through CTSD/Bid/Bax/Cytochrome C/caspase 9/caspase 3 pathway.

In contrary to its proapoptotic roles, CTSD has also been shown overexpressed and hypersecreted in numerous cancer types. Opposing roles of CTSD have been reported in cancer progression, metastasis and prognosis. The contradictive findings reflect the complex nature of CTSD gene and protein [23]. First, the CTSD gene promoter contains elements that confer both properties of a house-keeping gene and features of a regulated gene [24]. Second, the CTSD protein has three different molecular forms [25]. It is synthesized as a single chain pre-pro-cathepsin, which undergoes proteolytic cleavages to produce the active single chain pro-CTSD and finally the mature two-chain enzyme. Only the mature form of CTSD is enzymatically active. However, the enzyme-inactive form of pro-CTSD is abundantly present. While the nonenzymatic roles of CTSD still need to be fully investigated, CTSD is certainly not limited to degrading unwanted proteins but is involved in connecting other cellular processes. Third, CTSD has different subcellular localizations. CTSD is posttranscriptionally modified and processed along the route from the endoplasmic reticulum through the Golgi to its destination lysosome. Under certain conditions, pro-CTSD and mature CTSD can escape from lysosomes that provide the acidic condition required for activities of the enzyme. CTSD in cancer cells has an altered subcellular localization and an elevated secretion. Obviously, functions of misplaced CTSD can be distinct from its normal protease activity. Fourth, CTSD has multiple binding partners that regulate cell proliferation, differentiation and apoptosis. Therefore, the role of CTSD in cancer may be context dependent [21].

Conclusion

In summary, the novel bis-aryl urea compound N69B shows antiproliferative activities against multiple types of human and murine cancer cells through a distinct mechanism. While further studies are certainly needed for optimizing chemical structure and understanding full aspects of the mechanism, targeting CTSD may be a promising new approach in cancer drug development.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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