

Specific RSK Kinase Inhibition by Dibenzyl Trisulfide and Implication for Therapeutic Treatment of Cancer

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Abstract. *Background/Aim: The Jamaican “Guinea Hen Weed” (Petiveria alliacea L.) plant has been traditionally used in folklore medicine to treat a variety of diseases including cancer. In the present study we investigated on the therapeutic feasibility of dibenzyl trisulfide (DTS) (isolated from the Jamaican Guinea Hen Weed) as a potent small-molecule kinase inhibitor to treat cancer. Materials and Methods: We investigated the inhibitory effects of DTS against a large panel of kinases using a well-established competitive binding assay. Cell proliferation data were obtained using the WST-1 colorimetric assay. Results: DTS inhibited the activity of the C-terminal kinase domain of RSK1 (80% compared to control) with a K_d of 1.3 μM. Anti-proliferative effects of DTS were observed in small lung, pancreatic, breast, and prostate cancer cells with IC₅₀ values ranging from 0.34-0.84 μM. Conclusion: We have identified DTS as a highly selective and isoform-specific RSK1 kinase inhibitor with broad cancer therapeutic potential.*

Cancer is the leading cause of death by disease in Jamaica, with a life-time risk of 1 in 4 for males and 1 in 6 for females; prostate and breast being the most prevalent and deadly (1). The lack of proper screening methods and treatment facilities poses a major hindrance in the effort to reduce this cancer epidemic. Jamaica is known for its rich biodiversity and usage of medicinal plants for treating ailments of all kinds, and major efforts are underway to blend tradition with modern biomedical science.

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Approximately 40% of medications prescribed and used today are largely composed of compounds derived from medicinal plants. The sub-tropical shrub, *Petiveria alliacea* L., commonly known in Jamaica as the “Guinea Hen Weed”, has certain medicinal value. It is traditionally used in folk medicine to enhance memory and in the treatment of the common cold, flu, other viral or bacterial infections, inflammation, diabetes, and cancer.

Isolation of the immunomodulatory compound, dibenzyl trisulfide (DTS) (Figure 1) (2), and closer investigation of its biological activity, revealed its involvement in the mitogen-activated protein kinase (MAPK) signal transduction pathway (3). The MAPK pathway orchestrates a series of phosphorylation events essential for the proper regulation of embryogenesis, cell differentiation, cell proliferation, and cell death (4). Dysregulation of the pathway has been implicated in the pathogenesis of several human degenerative diseases and a variety of cancers (5). In neuroblastoma cells and human lung fibroblasts, DTS causes disassembly of microtubules, resulting in an anti-mitotic effect and subsequent inhibition of cell proliferation (3). The anti-proliferative and/or cytotoxic effects of DTS on several human cancer cell lines (SH-SY5Y neuroblastoma, MCF7 and M231 breast, IPC melanoma, A549 small cell lung cancer, A637 primary bladder carcinoma, Jurkat leukemia, A2780 and OVCAR4 ovarian, HT1080 fibrosarcoma, H460 non-small cell lung cancer, HeLa adenocarcinoma, and TE-671 sarcoma) have been presented (6).

It is estimated that approximately 30% of pharmaceutical industry research currently focuses on the study of kinases and their regulation, foreseeing a strong potential for therapeutic benefit (7). The feasibility of targeting protein kinases in cancer therapy with examples of inhibitors in clinical use are discussed in a review article by Pearson and Fabbro (8). Emerging roles for the RSK (p90 ribosomal s6 kinase) family of serine/threonine protein kinases, the most downstream effectors of the MAPK/ERK (Ras-extracellular signal regulated kinase) pathway, have recently been reported (9). Of particular

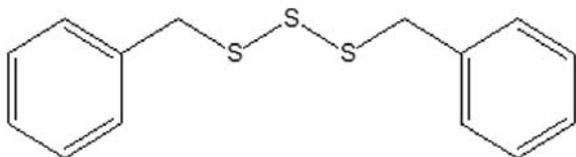


Figure 1. Chemical structure of dibenzyl trisulfide (DTS).

interest to our research group, is the pro-survival role of RSK in cancer. RSKs promote tumorigenesis in a variety of hormone-dependent and -independent cancers and its specific inhibition represents a promising therapeutic strategy (10).

Estrogen receptor- α (ER α) is a known target of RSK (11) and elevated levels of RSK are observed in various cancers (12, 13). Inhibition of RSK using the botanically-derived compound, SL0101, prevents breast cancer cell proliferation (14) and attenuates prostate cancer cell growth (13). Although RSK2 is most closely linked to human cancers (15), other isoforms also demonstrate anticancer activity. High protein expression of RSK3 and RSK4 in breast cancer cells confers resistance to apoptosis induced by PI3K/mTOR pathway inhibitors; however, resistance is overcome by combining PI3K/mTOR inhibitors and MEK (MAP/ERK) kinase inhibitor or pan-RSK kinase inhibitor (12). Surprisingly, MEK 1/2 inhibitors are still largely being used to study the physiological role of RSK (16). Unfortunately, therapeutic targeting of upstream components or “global regulators” of the MAP/ERK signaling pathway (i.e. MEK and Raf) causes significant patient side-effects and has so far been unsuccessful in the clinic (17). Therefore, more selective RSK inhibitors are required to study the specific function of RSK proteins. To date, there are only 3 inhibitors used to study the physiological function of RSKs however, there are no RSK isoform-specific inhibitors. Here we report, the isoform-specific inhibitory effect of DTS on the C-terminal kinase domain (CTKD) of RSK-1.

Materials and Methods

Drug compound. Dibenzyl Trisulfide (DTS) was purchased from International Laboratory USA (Lot No: 349813) and was prepared as a 10-mM stock solution in DMSO. A single final concentration of 10 μ M was used for the kinase inhibition assay in the primary screen.

Kinase inhibition assay. Using the KINOMEScan *in vitro* competition binding assay methods previously described (18-20), a primary screening of the DTS compound against a panel of 451 kinases was performed. Quantitative binding constants (K_d) of the best kinase-compound interactions were then determined as a secondary screening using an 11-dose response curve using the Hill equation. Curves were determined using a non-linear least square fit using the Levenberg-Marquardt algorithm.

Cell culturing and maintenance. All human cancer cell lines (A549, MDA-MB-231, Miapaca, MCF7, DU145, and PC-3) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were maintained in minimum essential media supplemented with 10% fetal calf serum, 1% L-glutamine, 2% penicillin-streptomycin, and 0.2% gentamicin.

Cell proliferation assay. The WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) (Roche) colorimetric assay was used to assess the anti-proliferative effect of DTS (21). Cells were trypsinized and plated into 96-well plates in 50 μ l of media and incubated overnight. Approximately 18 h after plating, 50 μ l of media was aspirated from each well and replaced with 50 μ l of media containing the required drug concentration. Cells were plated at a density to initiate a 72 h post-drug log phase of ~500-2000 cells/well. The DTS compound was solubilized in DMSO at 10-fold dilutions starting with 1 nM-100 μ M. The cells were then allowed to proliferate for 72 h at 37°C in humidified atmosphere of 5% CO₂. The experiment was terminated using 10 μ l per well WST-1 (Roche) and absorbance determined at 450 nm/690 nm. The effect of DTS on cell proliferation was measured as a percent of cell viability compared to control. The IC₅₀ values were determined using Graphpad Prism software. All experiments were carried out in duplicate and the mean results determined.

Results

Isolation of the small DTS molecule (Figure 1) from the Jamaican Guinea Hen Weed plant (2), the discovery of its involvement in MAPK signaling (3), and implications for its broad therapeutic potential (6, 22), sparked our interest to further study its role in kinase activity and cancer. Kinase inhibition by DTS (10 μ M) was determined using an *in vitro* competitive binding assay (18-20). Out of 451 kinases examined in the primary screening, only four were inhibited by more than 50%. The kinase identified with the highest level of inhibition was RSK1 (CTKD) at 80%. The binding affinities (K_d) of these top 4 kinases inhibited by DTS, were 1.3 μ M for RSK1 (CTKD) and >30 μ M for HUNK, PLK4, PIP5K2C (Table I). Furthermore, the inhibitory effect of DTS is highly selective. Out of 4 known isoforms of RSK (RSK1-4), only the CTKD of RSK1 is inhibited by DTS significantly (>65% is considered significant) (Table II).

To determine the anti-proliferative activity of DTS, we examined the integrity of 6 human cancer cell lines: A549 (small lung cancer), Miapaca (pancreatic cancer), MDA-MB-231 and MCF7 (breast cancer), and DU145 and PC-3 (prostate cancer) treated with DTS for 72 h (Figure 2). The current batch of DTS used in this study exhibited significant cytotoxic activity against 5 of the 6 cells lines, only ineffective against MCF-7 cells (Figure 2A). DTS was most effective (determined by IC₅₀ values) against Miapaca (0.34 μ M) and MDA-MB-231 (0.38 μ M) followed by DU145 (0.59 μ M), PC-3 (0.63 μ M), and A549 (0.84 μ M). Previous studies indicate IC₅₀ values (using different methods) that are also in keeping with the

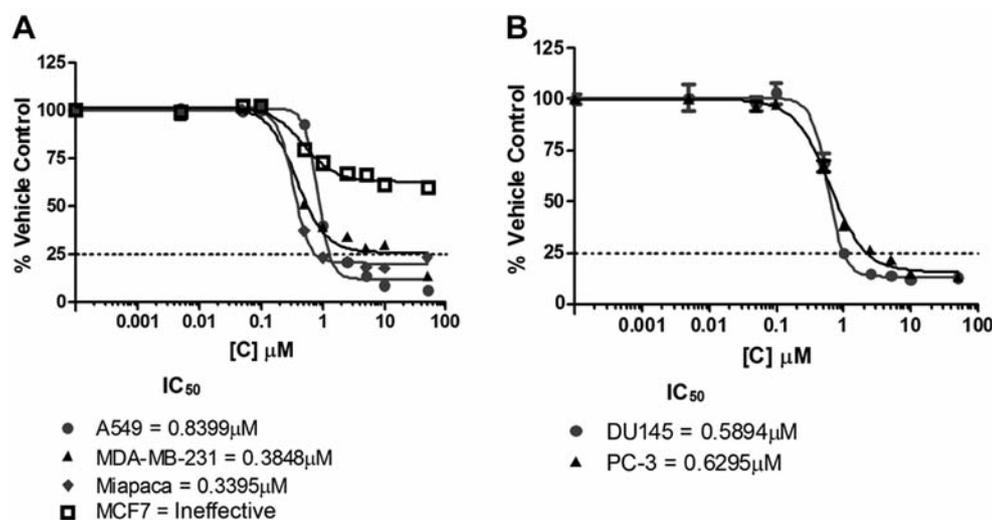


Figure 2. Cytotoxic effects of DTS on small lung, breast, pancreatic, and prostate cancer cells. Cells were plated into 96-well plates and dosed with DTS at 10-fold dilutions (1 nM to 100 μ M) for 72 h. Cell proliferation was measured using the WST-1 assay (Roche) according to the manufacturer's instructions. Results were graphed and IC₅₀ values were calculated using the Graph Pad software. Results for A549 (small lung), MDA-MB-231 and MCF7 (breast), Miapaca (pancreatic) are shown in (A). Results for prostate cancer cell lines (DU-145 and PC-3) are shown in B. Experiments were performed in duplicate and mean values were determined.

Table I. Kinases presenting an at least 50% inhibition in the primary kinase screening.

KINOMEscan Gene Symbol	% Control measured	% Inhibition	K _d (μ M)
<i>RSK1</i> (CTKD) ^a	20	80	1.3
<i>HUNK</i>	37	63	>30
<i>PLK4</i>	44	66	>30
<i>PIP5K2C</i>	45	55	>30

Results of the primary screen binding interactions are reported as % control, calculated as (test compound signal – positive control signal) / (negative control signal – positive control signal) multiplied by 100. % Inhibition is calculated as 100 - % control. ^aCTKD: C-terminal kinase domain.

cytotoxic effects of dibenzyl trisulfide (23-25) and other trisulfide derivatives (22) on various cancer cell lines however, with less potency than what is reported here.

Discussion

RSK kinase proteins are the molecules most further downstream of the classical Ras/MAPK/ERK signaling cascade and are canonically-activated by ERK 1/2 and PDK1. RSKs are characterized by two functional domains,

Table II. Comparison of the inhibitory effect of DTS on RSK kinases: Specific affinity for the C-terminal kinase domain of *RSK1*.

KINOMEscan Gene symbol	% Control measured	% Inhibition	K _d (μ M)
<i>RSK1</i> (NTKD) ^a	85	15	-
<i>RSK1</i> (CTKD) ^b	20	80	1.3
<i>RSK2</i> (NTKD)	100	0	-
<i>RSK2</i> (CTKD)	67	33	-
<i>RSK3</i> (NTKD)	91	9	-
<i>RSK3</i> (CTKD)	100	0	-
<i>RSK4</i> (NTKD)	93	7	-
<i>RSK4</i> (CTKD)	71	29	-

Results of the primary screen binding interactions are reported as % control, calculated as (test compound signal – positive control signal) / (negative control signal – positive control signal) multiplied by 100. % Inhibition is calculated as 100 - %control. K_d values were not determined for kinases with less than 50% inhibition. ^aNTKD: N-terminal kinase domain; ^bCTKD: C-terminal kinase domain.

Table III. Comparison of known RSK inhibitors.

Inhibitor name	Source	Isoform inhibited	Mechanism
BI-D1870 (27)	Synthesized	RSK1-4	Reversible; ATP- competitive inhibitor of NTKD ^a
SL0101 (28)	Isolated from <i>Fosteronia refracta</i> plant	RSK1,2,3	Reversible; ATP- competitive inhibitor of NTKD
Z-VAD-FMK (7)	Synthesized	RSK1,2,4	Irreversible; ATP- competitive inhibitor of CTKD ^b
DTS	Isolated from <i>Petiveria alliacea</i> L plant	RSK1	Irreversible?; ATP-competitive inhibitor of CTKD

^aNTKD: N-terminal kinase domain; ^bCTKD:C-terminal kinase domain.

an N-terminal kinase domain (NTKD) and C-terminal kinase domain (CTKD), connected by a linker region (9). ERK 1/2 phosphorylates the CTKD which allows for auto-phosphorylation of the linker region, followed by PDK1 binding and phosphorylation of the activation loop in the NTKD (26). Therefore, there are two logical sites for RSK inhibition: the ATP-binding site in the NTKD and the ATP-binding site in the CTKD (15). Out of 3 RSK inhibitors discovered so far (Table III), z-VAD-FMK (FMK) is the only one to inhibit the CTKD of RSK (7). However, z-VAD-fmk is commonly used as a pan-caspase inhibitor, bringing to question the feasibility of its use as a specific inhibitor of RSK kinases-only. Although Bain *et al.* suggests using a combination of BI-D1870 (10 μ M) and SL0101 or FMK to further improve the efficacy of RSK kinase inhibition (7), none of the aforementioned inhibitors are isoform-specific. We have identified DTS as a novel isoform-specific inhibitor of the CTKD region of RSK1. The discovery and development of specific inhibitors is useful for the elucidation of the precise physiological role(s) of individual RSK isoforms. The integrity of the cell death signaling pathway as a consequence of RSK1 kinase inhibition by DTS is currently under investigation in our laboratory. Further studies are required to investigate the precise inhibitory mechanism at the molecular level and to compare the efficacy of DTS against other known RSK inhibitors.

Conclusion

RSK kinases have been identified as a promising target for cancer treatment (7, 16, 27, 28). We are the first to report the highly selective inhibitory effect of DTS on an isoform-

specific RSK kinase. In addition, the anti-proliferative activity of DTS on several human cancer cell lines including prostate, breast, pancreatic, and lung has been confirmed. DTS remains a promising molecule for development as a cancer treatment alone or in combination with other agents. It is believed that this small-molecule RSK kinase inhibitor will offer a much less toxic and efficacious alternative cancer treatment compared to conventional chemotherapeutic drugs.

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