RESEARCH COMMUNICATION

# Cytostatic *in vitro* Effects of DTCM-Glutarimide on Bladder Carcinoma Cells

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## Abstract

**Bladder cancer is a common malignancy worldwide. Despite the increased use of cisplatin-based combination therapy, the outcomes for patients with advanced disease remain poor. Recently, altered activation of the PI3K/Akt/mTOR pathway has been associated with reduced patient survival and advanced stage of bladder cancer, making its upstream or downstream components attractive targets for therapeutic intervention. In the present study, we showed that treatment with DTCM-glutaramide, a piperidine that targets PDK1, results in reduced proliferation, diminished cell migration and G1 arrest in 5637 and T24 bladder carcinoma cells. Conversely, no apoptosis, necrosis or autophagy were detected after treatment, suggesting that reduced cell numbers *in vitro* are a result of diminished proliferation rather than cell death. Furthermore previous exposure to 10 μg/ml DTCM-glutarimide sensitized both cell lines to ionizing radiation. Although more studies are needed to corroborate our findings, our results indicate that PDK1 may be useful as a therapeutic target to prevent progression and abnormal tissue dissemination of urothelial carcinomas.**

**Keywords:** Urothelial cancer - PI3K/Akt/mTOR pathway - molecular target - therapy

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## Introduction

Carcinoma of the urinary bladder is a common cancer that accounts for approximately 300,000 new cases each year (Jemal et al., 2008). In the majority of patients the diagnosis is performed at early stages and treated with endoscopic and intravesical treatments. However, approximately 80% of patients recur in 1 to 2 years and despite radical cystectomy and systemic therapy, a substantial proportion of cases progress to invasive tumors and die from metastasis within 12-15 months (Kaufman, 2006).

In the last decade, efforts have been made in order identify clinically relevant biomarkers to assist tumor grading, prognosis, and prediction of treatment response. The abnormal activation of the PI3K/Akt/mTOR pathway has been repeatedly implicated in the development and maintenance of many human tumors, including urothelial carcinoma (Qian et al., 2009; Ching et al., 2010). The phosphatidylinositol 3-kinase PI3K is an essential enzyme in signal transduction from various stimuli to downstream pathways involved in diverse responses associated with growth, proliferation and survival. Activated PI3K generates phosphatidylinositol-3,4,5-triphosphate, which recruits phosphatidylinositol-dependent kinase 1 (PDK1) and Akt serine/threonine kinase at the plasma membrane, resulting in activation of Akt which in turn activates multiple downstream targets, including the mammalian target of rapamycin mTOR (Cully et al., 2006). Altered mTOR pathway activity is associated with reduced patient survival and advanced stage in bladder carcinoma (Hansel et al., 2010; Sun et al., 2011), therefore, the PDK1/Akt pathway is believed to be an attractive target for therapeutic intervention.

Here, we present evidence of the antiproliferative effects of the piperidine compound DTCM-glutarimide on 5637 and T24 cells offering a rationale for a therapeutic targeting in bladder cancer.

## Materials and Methods

### *Cell Culture*

The established bladder carcinoma cell lines 5637 (moderately differentiated tumour) and T24 (high-grade tumour) were obtained from the Cell Bank of the Federal University of Rio de Janeiro, Brazil. Cells were cultured

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in HAM F10 (T24) and RPMI 1640 (5637) (GibcoBRL, Life Technologies®, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100U/mL) and streptomycin (100ug/mL) at 37°C in a humidified 5% $CO_2$ incubator.

*Drug and treatments*

DTCM-glutarimide (3-[(dodecylthiocarbonyl) methyl]glutarimide) was synthesized as described earlier (Ishikawa et al., 2009). In one hand, this drug was designed and synthesized as a derivative of 9-methylstreptimidone that inhibits NF-kB (Wang et al., 2006). However, DTCM-gltarimide did not directly inhibit NF-kB. Rather, it exerted anti-inflammatory action by inhibiting AP-1 activity (Takeiri et al., 2011), while, new evidence has demonstrated its anti-tumoral effects by inhibiting PDK1 (Ito et al., 2011). For all experiments, cells were treated with 2.5, 5, 10 and 20 $\mu$g/ml. Corresponding control cultures received an equal volume of solvent.

*Measurement of cell growth*

Cell survival was assessed using the XTT assay (XTT II; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, equal amounts of cells were seeded in 96-well flat-bottom plates (2,500 cells/well) and allowed to attach to the incubator and proliferate for 24h. Subsequently, cells were treated with different concentrations of DTCM-g (2.5, 5, 10 and 20 $\mu$g/ml) and incubated for 24, 48 and 72h. After treatment, the culture medium was removed and replaced with medium containing 10 µL of XTT dye (3 mg/mL) in each well. The plates were incubated for 2 hours at 37°C and the formazan product was measured at 455 and 650 nm by using an iMarkmicroplate reader (Bio-Rad Laboratories®). For growth analyses, trypan blue exclusion and cell counting were also implemented. Each experiment was performed in triplicate wells and repeated in three sets of tests.

*Colony Formation Assay*

Clonogenic assays were performed according to Franken et al. (2006). After tripsinization, single cell suspensions of 300 cells were seeded in 6-well plates and treated with DTCM-g 2.5, 5, 10 and 20 $\mu$g/ml concentrations for 24h. Following that period, the culture medium was removed and replaced with drug-free medium. The cell cultures were incubated for 10 days and then the colonies were rinsed with PBS, fixed with methanol and stained with Giemsa. Only colonies with > 50 cells were counted. Assays were performed in triplicate.

*Cell cycle analysis*

In each time point after drug treatment, the cells were detached using trypsin-EDTA, fixed in 70% ethanol, stained with propidium iodide, and analysed on a Guava Personal Cell Analysis system (Guava Technologies, Hayward, CA, USA) according to the standard protocol provided by the manufacturer.

Percentages of cells in G0/G1, S, or G2/M phase were collected and processed using the GUAVA Cytosoft 4.2.1 version Software. All cell cycle analyses were performed in triplicate.

*Analysis of caspase activation*

For apoptosis $3x10^4$ cells were seeded on 6-well plates containing 3 ml of culture medium. After 24 hours, the medium was replaced and cells treated with the different concentrations of DTCM-g or vehicle only and cultured for additional 48h. Caspase activation was determined using the NucView™ 488 Caspase-3 Detection in Living Cells kit (Biotium Inc. Hayward, CA, USA) according to the manufacturer's instructions. Concisely, treated cells were trypsinized and incubated for 40 minutes at room temperature with the capase-3 substrate. After that period, cells were fixed in formaldehyde and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Then, samples were mounted, coverslipped and analysed by fluorescence microscopy with a triple filter. Five hundred nuclei were analysed per treatment and cells were scored and categorized according to differential staining.

*Detection of necrotic cells by differential staining*

Necrotic cells were recognized by differential staining according to Lee and Shacter (1999). Treated cells were trypsinized, centrifuged and incubated for 5 min at 37°C with bisbenzimide (Hoechst 33342), propidium iodide and fluorescein diacetate (Sigma Chemical Co., St. Louis, USA). Then, samples were mounted, coverslipped and analysed by fluorescence microscopy with a triple filter. Cells were scored and categorized as follows: (1) normal: blue nucleus and green cytoplasm, (2) apoptotic: fragmented blue nucleus and green cytoplasm and (3) necrotic: red nucleus. Five hundred nuclei were analysed per treatment.

*Detection and quantification of autophagy by acridine orange staining*

One of the most frequently used methods for following the autophagic process is staining with acidotropic dyes such as acridine orange (AO) (Klionsky et al., 2008). In AO-stained cells, the cytoplasm and nucleolus fluoresce bright green, whereas acidic autophagic vacuoles (autophagosomes) fluoresce bright red. After different treatments for 48h, cells were stained with AO at a final concentration of 1 µg/ml for 15 minutes, removed from the plate and collected in phenol red-free growth medium. Green and red fluorescence emission were measured with flow cytometry and the data were analyzed with the GUAVA Cytosoft 4.2.1 version Software.

*In vitro scratch assay for analysis of cell migration*

*In vitro* scratch assays to quantify tumour migration rates were performed according to Liang et al. (2007) with slight modifications. Briefly cells were grown to confluence on 12 well plates and scratch wounds were then created using a pipet tip (200$\mu$l) and the wound site photographed digitally at time zero. Cells were then treated with different concentrations of DTCM-g and subcultured for 24hs in medium supplemented with only 1% fetal bovine serum. After that period, cells were photographed and the Motic Images Plus v2.0 software (Motic China Group Co., Ltd) was used to calculate the cell-free area. Cell migration rate was calculated as the distance (nanometers) travelled by cells over time.

*Cell irradiation*

To test the effect of DTCM-g on radioresistance a proliferation-based assay (XTT assay) was used, which is highly comparable to the clonogenic assay when the cells are allowed to undergo 6 cell divisions (Pauwels et al., 2003). Cell cultures were irradiated with γ-rays from 60Cobalt at a dose rate of about 0.48Gy/min, using a Gammatron S-80 equipment (Siemens Medical Systems Inc., Iselin, NJ, USA) at the University Clinical Hospital (FMRP-USP). After irradiation with 2 and 4 Gy, the cells were plated in 96-well plates (100 microliters cell suspension, 500 cells/well) and the number of living cells was determined after 7 days by the proliferation XTT assay as described above.

The radiation dose enhancement ratio (DER) by DTCM-g was calculated using the following formula: DER = (surviving fraction at an indicated dose of radiation alone)/(surviving fraction at an indicated dose of radiation + DTCM-g). Dose enhancement ratio=1 suggests an additive radiation effect and DER>1, a supra-additive effect as against a sub-additive effect in the case of DER<1 (Tao et al., 2007).

*Statistical analysis*

Statistical analyses were performed by using the SigmaStat software (Jandel Scientific Company, San Rafael, CA, USA). One Way Repeated Measures Analysis of Variance (ANOVA) followed by the Holm-Sidak Pairwise Multiple Comparison was used to establish whether significant differences existed between groups. All tests were carried out for $\alpha = 0.05$.

## Results

*DTCM-g impairs cell proliferation in vitro*

DTCM-g effectively reduced the growth of 5637 cells after 48h at 20 $\mu$g/ml ($p<0.05$) when compared to control (DMSO 0.1%). Similarly, T24 cells showed significant growth reduction after 48h for DTCM-g 10 and 20 $\mu$g/ml. IC50 values were determined as 14.29 and 10.06 $\mu$g/ml for 5637 and T24, respectively, after 48hs of treatment. After 72h, both cell lines presented reduced growth at all concentrations tested ($p<0.05$) (Figure 1A), reducing the amount of living cells in the cultures by 47 and 53% for 5637 and T24, respectively (Figure 1A). Cell growth monitored at selected intervals by trypan blue exclusion showed a marked reduction of viable cells (Figure 1B).

*DTCM-g reduces the clonogenic capacity of T24 cells*

DTCM-g significantly reduced the colony formation capacity of the 5637 cell line at all concentrations tested when compared to control ($p<0.05$), reducing 68% of

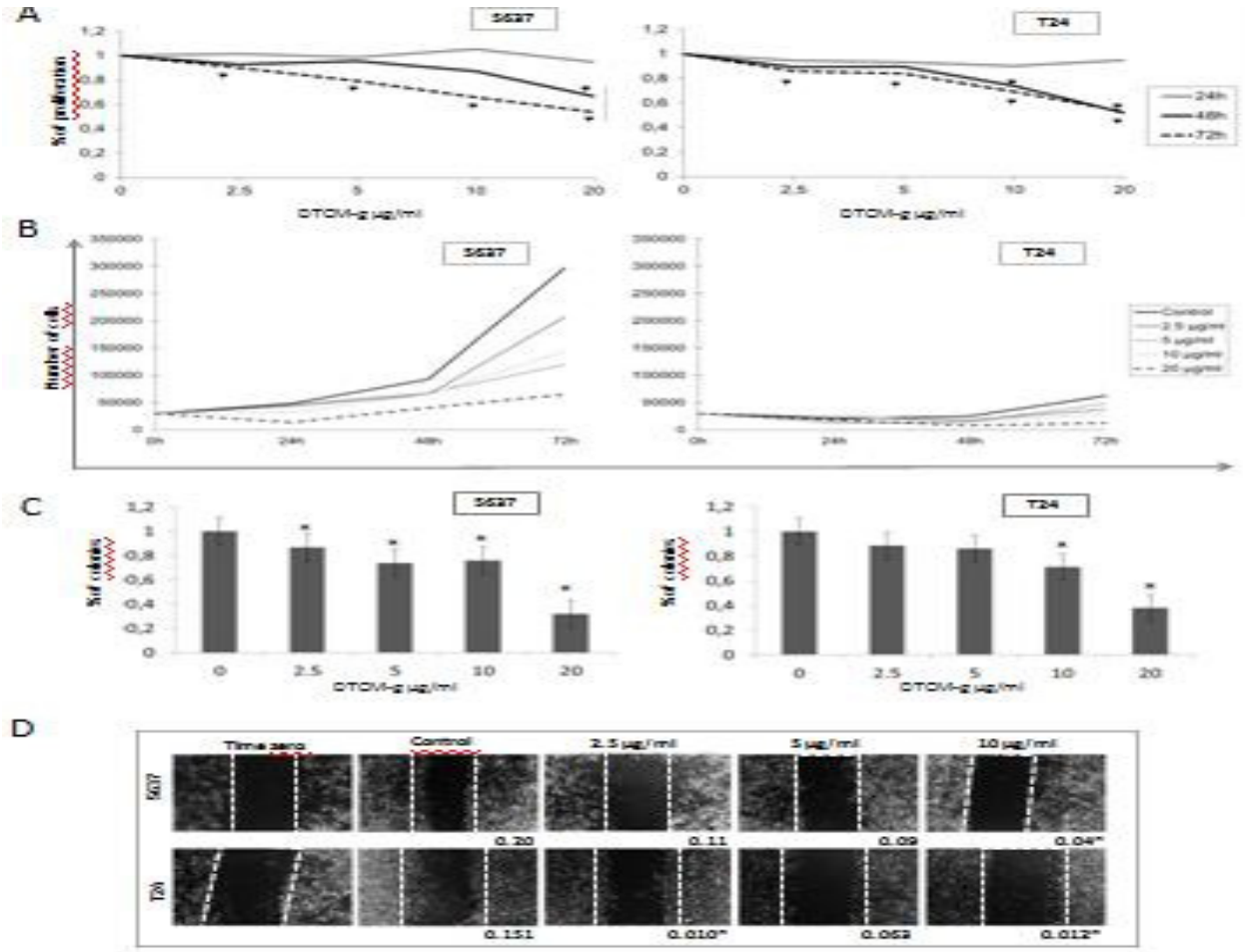


**Figure 1. Characterization of the Effects of PDK1 Inhibition on Cell Growth and Clonogenic Survival in 5637 and T24 Cells Treated with DTCM-g.** A) Growth inhibition in 5637 and T24 cell lines treated with DTCM-g at the indicated concentrations for 24h, 48h and 72h (XTT assay); B) Cell growth curve after treatment with the drug; C) DTCM-g inhibited the clonogenic capacity of both cell lines; D) DTCM-g treatments inhibited *in vitro* 5637 and T24 cell migration. Images were taken immediately and at 24 hours after wounding, in the absence (control) and presence of drug, as indicated. * Statistically different $p<0.05$.

**Table 1. Cell Cycle Analysis of Bladder Carcinoma Cell Lines Treated with DTCM-g. Percentages of Cells in G1, S, and G2/M Phases are Expressed as Mean ± Standard Deviation of Triplicates**.

| | | 5637 | | | T24 | | |
|---|---|---|---|---|---|---|---|
| | | 24h | 48h | 72h | 24h | 48h | 72h |
| Control | | 44.92 ± 1.38 | 54.30 ± 2.61 | 52.98 ± 3.16 | 43.82 ± 2.60 | 45.33 ± 1.45 | 48.56 ± 1.07 |
| G1 (%): | 2.5 μg/ml | 46.21 ± 1.22 | 56.79 ± 2.72 | 60.34 ± 4.71 | 50.62 ± 4.38 | 47.37 ± 1.23 | **51.41 ± 2.09** |
| | 5 μg/ml | 48.55 ± 2.32 | **58.82 ± 2.72** | 60.12 ± 6.44 | 50.95 ± 1.94 | 47.21 ± 1.82 | 47.57 ± 0.88 |
| | 10 μg/ml | **54.08 ± 2.56** | 57.65 ± 0.83 | **61.88 ± 0.76** | **57.87 ± 1.58** | **49.96 ± 5.08** | **45.90 ± 1.61** |
| | 20 μg/ml | **64.36 ± 2.04** | **64.32 ± 0.45** | 58.21 ± 2.16 | 46.36 ± 4.52 | 39.36 ± 6.28 | 30.79 ± 0.59 |
| Control | | 15.52 ± 2.73 | 14.27 ± 1.86 | 14.75 ± 4.65 | 17.08 ± 1.81 | 15.74 ± 1.29 | 15.13 ± 2.05 |
| S (%): | 2.5 μg/ml | 13.65 ± 1.07 | 14.30 ± 0.75 | 13.47 ± 2.68 | 16.14 ± 0.11 | 17.62 ± 0.96 | 13.80 ± 0.91 |
| | 5 μg/ml | 15.44 ± 1.90 | 12.27 ± 3.75 | 8.99 ± 0.15 | **12.44 ± 1.25** | 15.31 ± 1.53 | 15.17 ± 1.32 |
| | 10 μg/ml | 15.28 ± 1.12 | 14.47 ± 0.62 | 10.28 ± 1.36 | **9.72 ± 0.55** | 15.84 ± 2.68 | 14.31 ± 0.96 |
| | 20 μg/ml | **8.76 ± 0.41** | 10.49 ± 0.66 | 9.86 ± 1.19 | **7.66 ± 1.52** | 12.60 ± 2.63 | **8.14 ± 1.79** |
| Control | | 39.48 ± 1.04 | 30.13 ± 0.63 | 30.11 ± 3.40 | 23.18 ± 1.51 | 27.90 ± 3.23 | 24.28 ± 1.73 |
| G2/M (%): | 2.5 μg/ml | 38.57 ± 2.26 | 28.76 ± 1.88 | 25.82 ± 4.94 | 24.70 ± 2.45 | 27.07 ± 1.79 | 22.39 ± 1.14 |
| | 5 μg/ml | **34.49 ± 0.88** | 28.44 ± 5.28 | 29.51 ± 4.52 | 22.30 ± 1.38 | 28.81 ± 1.51 | 21.16 ± 0.72 |
| | 10 μg/ml | **29.04 ± 1.90** | 27.17 ± 0.68 | 27.21 ± 1.22 | 19.30 ± 2.49 | 23.52 ± 6.50 | **19.23 ± 1.41** |
| | 20 μg/ml | **14.01 ± 2.93** | **22.00 ± 0.65** | 28.29 ± 3.32 | **13.74 ± 0.58** | **18.23 ± 1.09** | **14.07 ± 2.70** |

**Table 2. Radiosensitization Induced by DTCM-g on 5637 and T24 Cell Lines. Cells were Pre-Treated with 10 μg/ml for 24h and Irradiated with 2 and 4 Gy. After 7 days Survival Fractions were Calculated**

| | Cell line | |
|---|---|---|
| | 5637 | T24 |
| 2 Gy | 1.00 | 1.25 |
| 4 Gy | 1.12 | 1.21 |

*DER = dose enhancement rate, DER=1 and DER>1 indicate additive and supra-additive effects, respectively

clonogenicity at the highest concentration. Similarly, the clonogenic capacity of T24 cell line was reduced in 29 and 63% at the 10 and 20 μg/ml treatments, respectively (Figure 1C).

*DTCM-g induces cell cycle arrest but not cell death in bladder carcinoma cell lines*

Treatment of the cells with DTCM-g induced a sustained change in the cell cycle distribution at all times tested. During each period, treated cells significantly accumulated in the G1 phase. The percentage of the cells in S and G2 phases decreased accordingly as a result of treatment while untreated cells (control) were more evenly distributed throughout the cell cycle (Table 1).

On the other hand, the analysis of cells treated with DTCM-g through caspase-3 activation showed no effect on apoptosis in this assay, while flow cytometry analysis failed to detect increased acidic vesicular organelles through AO staining. Comparatively, the microscopical analysis of treated cells by differential staining with propidium iodide was also unable to demonstrate higher frequency of necrotic-like cells for both cell lines, suggesting that reduced cell numbers *in vitro* result from impaired proliferation rather than cell death (data not shown).

*DTCM-g inhibits cell migration*

DTCM-g significantly reduced 5637 cell migration as measured by *in vitro* wound healing assays at 10 μg/ml ($p<0.05$). For T24 cells, migration was significantly inhibited after treatment with 2.5 and 10 μg/ml (Figure. 1D).

*DTCM-g pre-treatment sensitizes cells to ionizing radiation*

To study the cytotoxic effects of DTCM-g in association with γ-radiation, 5637 and T24 cells were incubated with 10 μg/ml of the drug for 24h. After treatment, the cell culture medium was replaced and cells irradiated with final doses of 2 and 4 Gy. The results showed that DTCM-g pre-treatment led to radiosensitization in both human bladder carcinoma cell lines at both doses tested (Table 2).

## Discussion

Over the past 30 years, systemic chemotherapy combining methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) has been the standard treatment option for patients with advanced or metastatic urothelial carcinoma. Nonetheless, a substantial proportion of patients relapse within the first year with a median survival of 12 months (Kaufman, 2006). Moreover, MVAC has shown to have significant toxicities including severe granulocytopenia, vomiting, and diarrhea (De Santis et al., 2009) and despite less toxic alternative treatment strategies, such as combinations of gemcitabine and cisplatin, the outcomes have not improved to any great extent. More recently, targeted therapies against metastatic or recurred urothelial carcinoma have emerged, mostly in the context of pre-clinical or phase I-II studies. Several molecular models directed to angiogenesis regulation and histone deacetylation are under study, some with promising results (Ismaelii et al., 2011).

The irregular activation of the PI3K /Akt / mTOR pathway has been involved in the oncogenesis of many tumors, including urothelial carcinoma (Qian et al., 2009). Several lines of evidence point to this pathway as an attractive target for cancer chemotherapy, mainly because it is relatively inactive in resting cells and it includes several upstream molecules (such as Ras and PI3K itself)

as well as many downstream targets of PI3K that can be aimed, including Akt and PDK1, among others (Sellers et al., 1999). Accordingly, in recent years, great interest in the search for small-molecule inhibitors that specifically inhibit key components of this pathway has emerged (Andersen et al., 2010). Several of these compounds show promising preclinical antitumor effects, and a few of them are currently undergoing clinical development (Peifer et al., 2009). Remarkably, much interest has been focused on PDK1. Only one single isoform of this protein has been so far reported in humans (Duronio, 2008), and unlike inhibitors of other members of the pathway, PDK1 inhibitors may reduce the activity of a large number of other growth-promoting kinases including Akt, S6K, SGK, PKC and RSK (Peifer et al., 2009).

In the present study, we demonstrate the antiproliferative effects of DTCM-g, a novel PDK1 inhibitor on two bladder carcinoma cell lines, 5637 and T24. Our results showed that DTCM-g significantly decreased cell proliferation, inducing G1 arrest. The half-maximal effective concentration at 48h was determined as 14.29 and 10.06 $\mu$g/ml for 5637 and T24 respectively, demonstrating DTCM-g effective action at low concentrations.

The potential anti-cancer effects of inhibiting the PDK1 have been previously assessed in other models. Sato et al., (2002) proved potent anti-tumor activity of the PDK1 inhibitor UCN-01 in murine colon adenocarcinoma NL-17 cells. Also, the inhibition of PDK1 by phenothiazines resulted in suppression of EGF-induced cell growth in ovary cancer cells and inhibited growth in other several cancer cell lines, including melanoma (SK-MEL-28), colon (HT29, Colo205, SW480, HCT116) and breast cancer (MCF7) (Choi et al., 2008). Likewise, the inhibitor OSU03012 had profound effects on medulloblastoma (Baryawno et al., 2010) and neuroblastoma survival *in vitro* and *in vivo* (Segerstrom et al., 2011). The PDK1 inhibitors, BX-795, BX-912, and BX-320 have also shown to inhibit growth in a wide panel of cell lines (Feldman et al., 2005).

Regarding bladder carcinoma, Hansel et al., (2010) demonstrated reduced cell proliferation when mTOR (a downstream target) was inhibited by rapamycin in RT4, T24, J82 and UMUC3 cell lines. Also in support of the *in vitro* data, treatment of mice bearing subcutaneous T24 xenografts resulted in a 55% reduction in tumor size. However, although T24 cells exhibit constitutive activation of the PI-3 kinase pathway (Wu et al., 2004), Hansel and co-worlkers (Hansel et al., 2010) were also unable to detect any apoptosis. Combinations of caspase-dependent and -independent mechanisms of cell death have been reported by others after PDK1 inhibition. While phenothiazines induced apoptosis in human ovary cancer cells (Choi et al., 2008), the PDK1 inhibitor OSU03012 induced mitochondrial dependent apoptosis of medulloblastoma cells (Baryawno et al., 2010), but was also reported to induce autophagy through accumulation of reactive oxygen species in hepatocellular carcinoma (Sargeant et al., 2007). In the present study, cytometric analysis after AO staining failed to detect increased autophagy in cells treated with DTCM-g. Also, we were unable to detect any necrosis after treating cells with this drug, suggesting that reduced cell numbers may result of impaired proliferation rather than cell death. On the other hand, flow cytometry analysis demonstrated sustained G1 arrest in 5637 and T24 cells after treatment with DTCM-g. Similarly, the dual PI3 kinase/mTOR inhibitor PI-103 induced a G1 arrest but no apoptosis in glioma (Fan et al., 2006) and G1 arrest with only minor apoptosis in neuroblastoma cells treated with the same inhibitor (Segerstrom et al., 2011).

Treatment with DTCM-g also demonstrated to sensitize 5637 and T24 cells to ionizing radiation. Although combined chemotherapy with radiation for the treatment of advanced bladder cancer still remains questionable, a recent study by Ikeda et al. (2011) demonstrated that radiotherapy in combination with MVAC offers a survival benefit, particularly in locally advanced or metastatic bladder cancers. Moreover, radiotherapy can be indicated for unresectable tumors, or in inoperable patients, when combination with chemotherapy is not possible (Marta et al., 2012). Previous reports have demonstrated the radiosensitizing effects of inhibiting the PI3/Akt pathway in human bladder carcinoma T24 cells (Kim et al., 2005). Regarding PDK1, our findings are in accord with those of Gupta et al. (2003) who showed that its inactivation by LY294002 resulted in significant and synergistic reduction in clonogenicity and growth delay in xenografted T24 cells when combined with radiation.

On the other hand, PI3/Akt pathway has demonstrated to be a critical downstream mediator of motility regulating invasion (Rieger-Christ et al., 2004). In the present study inhibition of PDK1 by DTCM-g significantly reduced migration as measured by *in vitro* wound healing assays in both aggressive bladder carcinoma cell lines, a relevant correlate to depth of invasion in human specimens. Similar results were obtained by Liu et al., (2009) who showed that small interference RNA markedly inhibited spontaneous migration and experimental metastasis of human breast cancer cells. Specially, Wu et al. (2004) also showed strong inhibition of invasion by the highly aggressive bladder tumor cell lines T24, J82 and UM-UC-3v treated with the PDK1 inhibitor LY294002.

Hence, even though more studies are needed to corroborate our findings, our results indicate that directing against PDK1 through DTCM-g treatment could be useful to prevent cancer progression and abnormal tissue dissemination of advanced bladder carcinoma.

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