Investigational New Drugs (2019) 37:779–783
https://doi.org/10.1007/s10637-019-00726-2

SHORT REPORT

Inhibition of AKT signalling by benzoxazine derivative LTUR6 through the modulation of downstream kinases

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Received: 15 November 2018 / Accepted: 4 January 2019 / Published online: 10 January 2019

Summary
Many compounds structurally similar to chromones have been developed to enhance the sensitizing effect of cancer cells to chemotherapeutic agents. Most of these compounds have been shown to promote this sensitization by targeting the repair pathways. One such compound is LTUR6, which enhances the sensitization of doxorubicin to colon cancer cells HT29, by inhibiting the phosphorylation of the double stranded break (DSB) repair enzyme AKT. The downstream regulatory targets of AKT that enhance doxorubicin mediated cytotoxicity in the presence of LTUR6 remains elusive. In this study, we performed comparative analyses of 43 kinase phosphorylation sites using the human phospho-kinase array proteome profiler. Results revealed altered expression levels of multiple proteins that regulated apoptotic signalling pathways. Increased activation of mTOR, RSK1/2/3, p38α and PRAS40 after combination treatment with LTUR6 and doxorubicin over doxorubicin alone was observed. This study provides a deeper insight into the key proteins involved and presents a novel molecular pathway.

Keywords Doxorubicin · Apoptosis · AKT · Chemosensitization

Introduction

PI3Ks are lipid kinases that play an important regulatory role in various cellular processes such as cell proliferation, cell cycle regulation, apoptosis, cell differentiation, DNA repair, senescence, angiogenesis and cellular metabolism. It is the most activated signal transduction pathway involved in pathological disorders such as cancer [1]. Downstream to receptor tyrosine kinases and G protein couple receptors, class I PI3Ks is the transformation of the 3-hydroxy 4,5-biphosphate (PIP2) to 3, 4, 5-tris-phosphate (PIP3) when activated [2]. In the appropriate cellular context, second messengers like PIP2 and PIP3 mediate diverse physiological processes including cell growth, survival, proliferation, chemotaxis and tumorigenesis [3]. The class 1 PI3Ks can be further divided into three class IA PI3Ks (α, β, δ) and one class IB PI3K (γ). The mechanism of signal transmission is through the recruitment of various substrates of the activated enzyme, present either in the cytosol or nucleus, to the plasma membrane where they are then able to dock to the phosphorylated lipids. For instance, following generation of secondary messengers (PIP2, PIP3), PI3K signalling impinges on a diverse array of intracellular signalling proteins such as the serine-threonine kinases (AKT, PDK1), protein tyrosine kinases (Tec, BTK family), cytoskeletal proteins, exchange factors for GTP-binding proteins (Grp1 and Rac factors) and adaptor proteins (GAB-1) [4, 5]. This can trigger a cascade of events that activates multiple effector kinase pathways such as ERK1/2, AKT, NF-kappa-B, BTK, PKC and JNK/SAPK pathways regulating the growth and survival of unperturbed cells [6].

Increasing the sensitivity of cancer cells to chemotherapy has been the focus of numerous studies. Targeting the DNA repair pathways of cancer cells has been a mechanism of great interest over the past decade, many inhibitors acting on this pathway has been developed and are currently in the clinical

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trial stage. PI3K family inhibitors, including DNA-PK inhibitors belonging to the class of chromones such as LY294002 and wortmannin, enhance the sensitization of cancer cells to chemo/radiotherapy by inhibition of DNA repair [7, 8]. However, their underlying mechanisms of action have seldom been unravelled and these compounds have been repeatedly shown to lack enzyme-specificity.

In our recent publication, we demonstrated that a benzoxazine analogue of the chromones, LTUR6 (compound no: 17a), is a potent and specific chemosensitizer and inhibits DSB repair due to its selective inhibition of PI3K-δ [9]. We also reported that LTUR6 in combination with Doxorubicin inhibits AKT, which plays a key role in response to DNA DSBs repair machinery [10]. However, the involvement of downstream targets of AKT in LTUR6-mediated chemosensitization was not elucidated. Hence, the present study was carried out to identify the important molecular determinants and the subsequent molecular pathways altered to enhance Doxorubicin mediated cytotoxicity in presence of LTUR6.

Materials and methods

Cell lines

Human colon tumour HT29 cells were purchased from American Type Culture Collection (ATCC, Maryland, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Scientific HyClone) containing 10% Fetal Bovine Serum (In Vitro Technologies, Auckland, New Zealand), 4.5 g glucose/L, 4 mM L-glutamine and sodium pyruvate. Culture was maintained in a humidified atmosphere at 37 °C with 5% CO_2.

Compounds and drugs used

Stock solution for LTUR6 (supplied by Dr. Jasim Al-Rawi and Rick Morrison, La Trobe University Bendigo, Australia) of 1 mM was prepared in DMSO and stored at −20 °C and further diluted in culture medium to obtain the required concentration. Doxorubicin, topoisomerase II inhibitor, was dissolved in Milli-Q H_2O and stored at −20 °C. A stock solution of 1 mM was prepared from which further dilutions were made in media.

Human phospho-kinase antibody array

The human phospho-kinase array was performed based on the manufacturer's protocol. In this array, 46 capture antibodies or control antibodies against human phosphorylated kinases were spotted in duplicate on nitrocellulose membranes. Before harvesting, cells were treated with control (DMSO), compound alone (LTUR6), Doxorubicin alone, or a combination of Doxorubicin and compounds. They were then lysed using a lysis buffer (ARY003, Proteome Profiler™, R&D Systems, Minneapolis, MN, USA) and the total protein concentration of each sample was quantified using the Bio-Rad DC protein assay kit II (Cat #500–0112, Bio-Rad Laboratories, Philadelphia). A concentration of 150 μg/ml was used. The lysates were incubated overnight in array membranes and after 24 h, they were washed to remove unbound proteins. Further incubation was performed with a cocktail of biotinylated detection antibodies for 2 h at room temperature. Membranes were

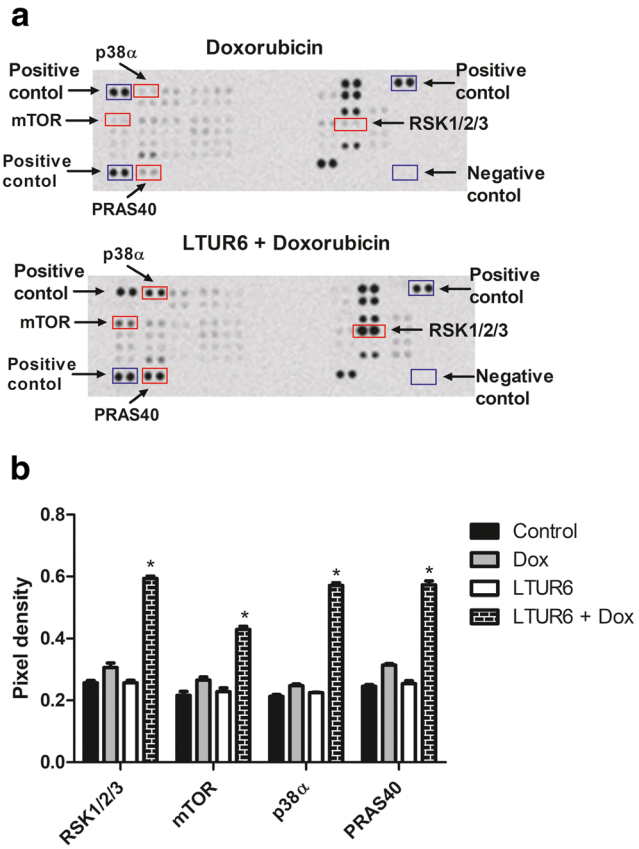

Fig. 1 Key proteins identified in regulating apoptotic pathway downstream to AKT. Pre-treatment with LTUR6 enhanced doxorubicin-mediated cytotoxicity by regulating apoptosis. HT-29 cell lysates were treated for 24 h with Doxorubicin alone and in combination with LTUR6. Proteins bound to the membrane were detected by exposing to an ECL chemiluminescent and images captured using Syngene G-BOX. **a** A representative phospho-kinase array panel showing levels of proteins in HT29 cell sample. The spots were scanned and digitized. Proteins expression levels quantified by measuring the pixel densities were plotted in the form of a bar diagram **b** showed increased activation over doxorubicin alone and combination. Graph indicates average of three replicates. Statistical significance was determined by one way ANOVA. * represents $p < 0.05$ for LTUR6 + doxorubicin relative to doxorubicin alone treated cells

then exposed to streptavidin-HRP for 30 min. After a final wash the proteins bound to the membrane were detected by exposing to an ECL chemiluminescent reagent for 1 min. Chemiluminescent images were captured using Syngene G-BOX (G: BOX-CHEMI-XL1.4, USA). For quantifying the activation levels of the proteins, the integrated optical density (IOD) of each spot was read at increasing exposures using a Bio-Rad ChemiDoc station. IOD values were corrected for background signal. For comparing different membranes, values were normalized to those of the positive controls on each membrane and the protein expression levels were then quantified. The experiment was performed twice to confirm the results obtained.

Statistical analyses

Data was analysed using GraphPad prism 5.0 and presented as mean ± SEM.

Results and discussion

Screening for activated kinases in HT-29 following chemosensitization with doxorubicin and LTUR6

Figure 1a shows a representation of the array membrane following treatment with doxorubicin and in combination with LTUR6 in HT29, respectively. Proteins that showed a change in activation have been highlighted. Dot intensity was quantified and graphically presented in Fig. 1b showing an increased activation of RSK1/2/3, mTOR, p38α and PRAS40 respectively, after combination treatment with LTUR6 and Doxorubicin over Doxorubicin alone.

Downstream targets of AKT signalling in apoptotic pathway

The findings from one of our previous studies showed that LTUR6 in combination with Doxorubicin inhibited AKT phosphorylation at Ser473 and Thr308 [10]. Having established a role for AKT, the next aim was to identify the different downstream targets that participate in the signalling of the particular apoptotic response pathways observed using human phosphokinase arrays and HT29 cells. Increased activation of RSK1/2/3, mTOR, p38α and PRAS40 after combination treatment with LTUR6 and doxorubicin over doxorubicin alone was observed. The RSKs are a family of serine/threonine kinases that respond to various stimuli, being activated almost exclusively by extracellular signal related kinases 1/2 (ERK 1/2) [11]. The RSK family consist of 4 isoforms denoted RSK 1/2/3/4, of which RSK 1/2/3 are most active. RSK1/2/3 activate many proteins involved in cell cycle regulation while inactivating many pro-apoptotic proteins. This promotes cell cycle progression and survival of a variety of cancer cell lines [12]. RSK is up regulated in many cancers, and development of inhibitor molecules against RSK have revealed opposing effects stimulated by the absence of RSK, including both the induction of apoptosis and induction of resistance to apoptosis, mediated through up regulation of p21 [13]. In the context of our results, the up regulation of RSK in the combination treatment, and the observed decrease in survival, could be due to the competing effects of p21. RSK also activated the mTORC1 complex [14], which is known to inhibit apoptosis, but in a complex feedback system including PRAS40 (described below) it promotes apoptosis, as was observed in the current system.

Additionally, there was an additive effect of Doxorubicin and LTUR6 on the phosphorylation of the

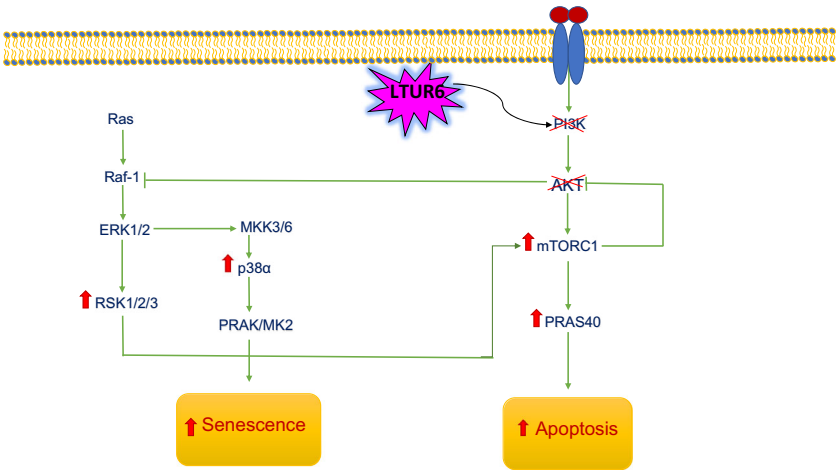

Fig. 2 Proposed mechanism of action LTUR6. LTUR6 appears to promote the downstream activation of RSK1/2/3, mTOR, p38 and PRAS40

mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription [15]. mTOR is activated by the PI3K/AKT signalling pathway [16], and integrates the input from upstream pathways including insulin, growth factors (such as IGF-1 and IGF-2), and amino acids; mTOR also senses cellular stresses via nutrient, oxygen, and energy levels. The mTOR pathway is dysregulated in human diseases such as diabetes, obesity, depression, and certain cancers [15]. The association between AKT and mTOR activity is complicated and a multi-step phenomenon. The main function of mTOR involves protein translation through phosphorylation of its downstream targets S6K, 4EBP1 and ULK1. The mTOR/Raptor/mLST8 complex (mTORC1) downstream of AKT, is also known to inhibit AKT via a negative feedback loop [17].

The proline-rich AKT-substrate-40 (PRAS40) is both a downstream target of mTORC1, and an inhibitor of mTORC1 signalling; in a negative feedback loop [18]. Interestingly, PRAS40 was also upregulated in the combination of Doxorubicin and LTUR6 and a recent study reported an mTORC1-independent pro-apoptotic function of PRAS40 [19]. In line with the above statements, increased activation of mTORC1 facilitates further inhibition of AKT. Activation of mTOR reduces apoptosis [19], whereas inhibition of mTORC1 by PRAS40 induces or facilitates apoptosis in several cell lines [20].

The final protein that showed increased phosphorylation in the combination treatment was p38α mitogen-activated protein kinases (p38α MAPK), a proline-directed Ser/Thr MAP kinase, and plays an important role in cellular responses to inflammatory cytokines, DNA damage, oxidative stress, replicative senescence, oncogene-induced senescence, DNA-damage responses, and contact-inhibition, which ultimately suppresses tumorigenesis [21]. AKT is known to negatively regulate the activity of p38α MAPK [22], so inhibition of AKT would lead to an increase in p38α MAPK activity as we have seen here. Recently, a study reported signalling between PI3K/AKT and p38α MAPK contributed to the balance of pro- versus anti-apoptotic signalling in endothelial cells. Inhibition of PI3K/AKT (10 μM LY294002) resulted in activation of p38α MAPK stimulating the pro-apoptotic pathways [21, 23]. Consistent with this notion, inhibition of PI3K/AKT by LTUR6, increased phosphorylation of p38α MAPK, and lead to an increase in apoptosis in the current study.

To recapitulate, this work proposes (Fig. 2) that the chemosensitization observed following inhibition of PI3K is likely due to the involvement several downstream targets of AKT, including RSK1/2/3, mTOR, p38 and PRAS40 which regulate cell cycle arrest, DNA repair and apoptosis.

Acknowledgments The authors would like to thank Dr. Jasim Al-Rawi and Rick Morrison for supplying LTUR6 compound, which was synthesized in their laboratory. The authors would like to thank Dr. Mike Angove for his constant encouragement and support.

Funding This work was funded by Australian Postgraduate Award and LaTrobe University Postgraduate Research Scholarship (PhD fellowship to Rejitha Suraj).

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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