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Effect of Alpha-1-Adrenoceptor Blocker on Cytosolic Enzyme Targets for Potential use in Cancer Chemotherapy

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Abstract: Doxazosin is one of the quinazoline-based alpha 1-adrenergic receptor antagonists in clinical use for the treatment of hypertension and benign prostate hyperplasia. Doxazosin-induced cytotoxicity studies, resulted in growth inhibition and apoptosis, show its potential therapeutic benefits for several forms of cancers. These effects on cells occur as adrenoceptor-independent mechanisms, as observed with other quinazoline family of alpha-1 blockers. Moreover, Doxazosin induced apoptosis is associated with pathways, including EGFR, NF-κβ and TGF-β signaling which typically engage Src as a central signaling component. Recent evidences show that glutathione transferases, may also contribute to these signaling events, through the kinases that share signaling pathways with Src, responsible for the regulation of transferase activity. In addition, the overactive glutathione transferases are related with anticancer drug resistance, as well as cancer development. Therefore, in the present study, the anticancer potential of Doxazosin was investigated by *in vitro* enzyme assays that were used to develop full dose-response profiles of drug at varying doses. The drug dose that exerts 50% inhibition of enzyme activity is defined as IC_{50} value and determined through the nonlinear regression analysis of dose-response data. The IC_{50} values determined for Src kinase, total protein tyrosine kinase, cytosolic total Src family kinase and total glutathione transferase enzymes were within nanomolar to low micromolar range. These results suggest that Doxazosin may be used to improve multifunctional therapeutic formulations to provide reduced drug resistance and enhanced cytotoxicity at target tissues.

Key words: Doxazosin mesylate, glutathione transferase, non-receptor tyrosine kinase, small molecule inhibitor, bovine liver cytosol

INTRODUCTION

Doxazosin is one of the quinazoline-based alpha 1-adrenergic receptor antagonists in clinical use for the treatment of hypertension and Benign Prostate Hyperplasia (BPH). Upon prolonged use, it was reported to cause the cardiac toxicity (Allhat, 2000) and heart failure risk through the apoptosis of cardiomyocytes. Later, apoptosis of prostate cancer cells was defined via significant loss in viability and directly related with the quinazoline nucleus of the drug (Kyprianou and Benning, 2000). This study revealed its potential therapeutic benefits for several forms of cancers by the induction of apoptosis and growth inhibition on the various human cancer cells, including prostate (Gonzalez-Juanatey *et al*., 2003). Later, this cytotoxic effect was associated with the presence of quinazoline nucleus, as observed with other quinazoline family of alpha-1 blockers (Alberti, 2007; Kyprianou *et al*., 2009). The mechanism was independent of alpha-1-adrenergic-receptor (adrenoceptor) involvement (Kyprianou and Jacobs, 2000; Kyprianou *et al*., 2009; Shaw *et al*., 2004) but engaging several other signaling components, such as Akt, FAS, EGFR, NF-κβ and TGF-β (Eiras *et al*., 2006; Hui *et al*., 2008; Keledjian *et al*., 2005; Walden *et al*., 2004). The common feature of these signaling components is to recruit active Src, a prototype member of Src family nonreceptor tyrosine kinase, in mechanisms they participate. Src and other members of Src family tyrosine kinases (SFKs) are fundamental constituents of the signaling processes from cell membrane to nucleus and SFK inhibitors are important to regulate the impaired function of the signal transduction (Guarino, 2010; Isgor and Isgor, 2011b; Mayer and Krop, 2010; Schenone *et al*., 2011). Quinazoline derivatives (I, Fig. 1) are known for decades as small molecule tyrosine kinase inhibitors and especially one of the most effective inhibitor types for SFKs (El-Azab *et al*., 2010; Wu *et al*.,

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2011). Several quinazoline derivatives were already patented as Src family kinase inhibitors and some of them in clinical studies to be develop as anticancer therapeutics (Pytel *et al*., 2009). Moreover, the effect of kinase inhibitor drugs with quinazoline core are still under study and several clinical studies (Clinicaltrial.gov) are still evaluating patients with solid tumors. Of the molecules with quinazoline core, gefitinib (ZD 1839) is the first selective inhibitor (II, Fig. 1) against the tyrosine kinase activity of EGFR for cancer chemotherapy (Muhsin *et al*., 2003) and currently available for the treatment of non-small cell lung cancer (Hatziagapiou *et al*., 2011; Srivastava *et al*., 2009; Teraishi *et al*., 2005). Saracatinib (AZD 0530), another Src kinase inhibitor (III, Fig. 1) were discovered to interrupt downstream of NFκβ signaling and hence, in advanced prostate cancers, cause the obstruction of Receptor Tyrosine Kinase (RTK) activity linked to metastasis and decrease in the proliferation rate (Yang *et al*., 2010). In small cell lung cancer, the presence of amino-thiazole inhibitor dasatinib (IV, Fig. 1) and pyrazolo[3,4-d] pyrimidine derivative PP2 (V, Fig. 1), a multiple and Src selective tyrosine kinase inhibitors, respectively, were shown to enhance the therapeutic effectiveness of topoisomerase II inhibitor amrubicin (Ueda *et al*., 2009). Dasatinib is still under study to evaluate possible benefits for various tumors, including cancers of pancreas and prostate (Lara *et al*., 2009). Although the studies with Doxazosin (VI, Fig. 1) are indicating its possible contribution to Src mediated signaling cascades, there is no study evaluating its effect on non-receptor tyrosine kinase activities associated with cancer (Schenone *et al*., 2011). In this context, Doxazosin and its novel derivatives were shown to induce apoptosis on human prostate cancer cell lines, partially through Akt inhibition. With this study, the role of quinazoline nucleus was verified (Shaw *et al*., 2004). The potential mechanisms identified as deregulation of signal transduction pathways relating TGF-β and anoikis which is the interruption of cell attachment to the extracellular matrix of prostate cancer cells (Kyprianou, 2003; Kyprianou and Jacobs, 2000; Kyprianou *et al*., 2009). The suppression of endothelial cell migration and antiangiogenic response was implicated in down regulation of VEGF expression and eventually Doxazosin interfering with VEGF and FGF-2 function in a dose dependent-manner (Keledjian *et al*., 2005). Doxazosin was found to induce apoptosis in prostate epithelial cells with the stimulation of TGF-β1 signaling by its effectors which are also the nuclear regulatory proteins for cell cycle progression (Partin *et al*., 2003). Another Doxazosin activated nuclear mechanism for prostate cell apoptosis was DNA-damage mediated apoptosis as shown with the microarray analysis of LNCaP, androgen-dependent human prostate cancer cell line (Arencibia *et al*., 2005). The underlying mechanism was associated with endoplasmic reticulum stress through C/EBPβ, p38 MAPK activated transcription factor GADD153 and FAK related mechanisms (Eiras *et al*., 2006), similar to the results with earlier studies on prostate cancer cells (Partin *et al*., 2003; Walden *et al*., 2004). For pituitary tumors, the apoptotic mechanism was partially independent of alpha-adrenergic receptor inhibition but engaging down-regulation of

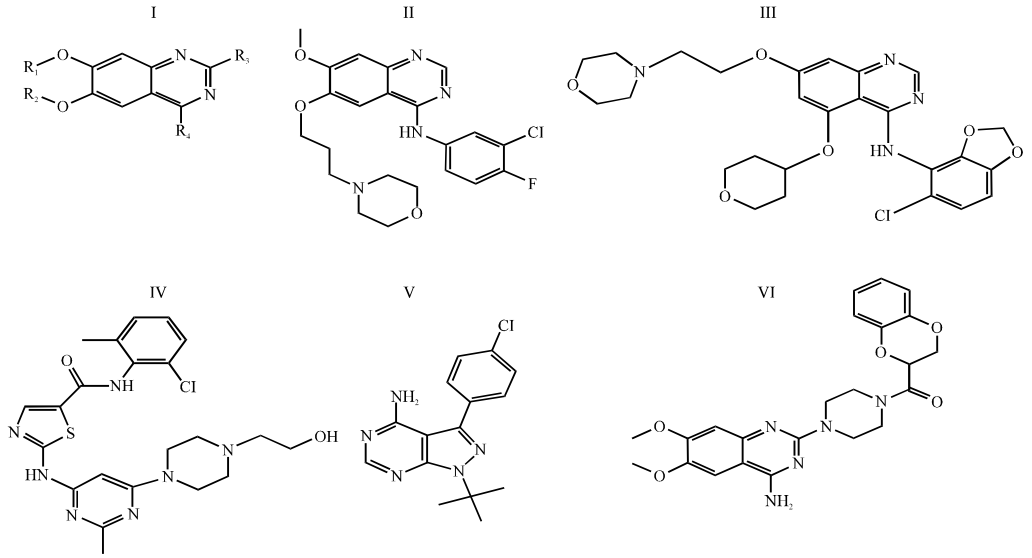

Fig. 1: Structures for (I) quinazoline derivative, (II) Gefitinib, (III) Saracatinib, (IV) Dasatinib, (V) PP2 and (VI) Doxazosin

NFκB signaling (Fernando and Heaney, 2005). Apoptosis induction with Doxazosin was also evaluated with breast and ovary cancer cell lines. For breast cancer cells, the mechanism found directly related with EGFR and NFκB signalling pathways (Hui *et al*., 2008), as well as association with transcription factor activator protein-2α (AP-2α). On ovary cancer cell line, the dominating mechanism was also reported as the alterations in AP-2α transcript levels (Anttila *et al*., 2000; Gan *et al*., 2008). Considering that AP-2α affects the cell viability by disturbing the function of many protein and transcription factors, thus, AP-2α-mediated apoptosis points out the nuclear processes as observed with prostate and ovarian cancer cells (Gan *et al*., 2008; Partin *et al*., 2003). For therapeutic purposes, devising small molecule drugs that interfere with the nuclear components seems invaluable tool to block the signaling from membrane to nucleus. But, the molecular interactions with DNA is under tight control provided by signaling components, such as kinases and also by detoxification system enzymes which are mostly the GSTs (Singh *et al*., 2010). Eventually, drugs targeting nuclear structures, such platinum drugs, are shown to induce resistance upon GST mediated detoxification reactions or GST linked signaling events (Fig. 1). Although Doxazosin was shown to affect components in nuclear mechanisms and it is verified that the bioavailable drug is first utilized in liver mainly by GSTs, as explained before, to our best knowledge, there is no report published yet to delineate the effect of Doxazosin on Src kinase and GSTs. In this current study, hence, in an effort to explore the possible off-targets of Doxazosin, we evaluated the biological activity of drug at varying doses on GST and Src enzymes which are important for therapeutic effectiveness of drugs in anticancer therapeutic development.

MATERIALS AND METHODS

The tyrosine kinase inhibitors Genistein and SU6656 were purchased from Sigma-Aldrich, St. Louis, MO, USA. Takara Universal Tyrosine Assay ELISA plates and reagents was purchased from Takara-Bio Inc., Shiga, Japan; recombinant c-Src tyrosine kinase and ProFluor fluorescent tyrosine kinase reagents were purchased from Promega, Madison, WI, USA. Bovine liver cytosolic fraction was used as GST enzyme source and prepared in our laboratory. Other chemicals were at analytical grade and purchased from Sigma-Aldrich, St. Louis, MO, USA. The kinetic and end-point detection to measure the substrate phosphorylation or glutathione (GSH) conjugation was performed with absorbance and fluorescence based assays and recorded by SpectraMax-M2e Multi-Mode Microplate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA. The enzyme calibration and the dose response curves were constructed using 3-4 independent experiments, each in duplicates. The construction of calibration plots, the dose-response curves and determining the 50% inhibitory concentration (IC_{50}) were achieved by non-linear regression analysis, using the four-parameter logistic equation (Sigmoidal dose-response, GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California USA).

Preparation of compounds: SU6656, Genistein and Ethacrynic acid was dissolved in DMSO and used in assays as a positive control. To accomplish maximum enzyme inhibition for corresponding targets, namely, SFKs, total PTK and GST, the final concentrations of SU6656, Genistein and Ethacrynic acid was 500 nM, 50 μM and 100 μM, respectively. (Akiyama *et al*., 1987; Blake *et al*., 2000; Ploemen *et al*., 1990). The Doxazosin stock solution was prepared at 4.86 mM from tablets containing 4 mg Doxazosin available in the drugstores. Briefly, the tablets grounded to fine powder were dissolved in PBS with continuous stirring for 24 h at room temperature. The insoluble material filtered off and the concentration was determined via spectrophotometric methods to verify the calculated final concentration for 20 tablets in PBS (pH 7) at λ_{max} of 256 nm (El-Sayed and El-Salem, 2005). Further dilutions were made from stock solution of Doxazosin in the concentration range of 0.010-250 μM. The stock solutions of inhibitors were prepared in DMSO and the dilutions were made with appropriate assay buffer, where the final concentration of dilutions did not exceed 0.5% DMSO.

Preparation of cytosol from bovine liver: The bovine liver cytosol were prepared from the fresh liver samples from well bled 6-12 months old cattle which were obtained from slaughter-house in Kazan, Ankara, Turkiye (Isgor *et al*., 2010). The liver samples, weighed 20-25 g, were homogenized in 10 mM potassium phosphate buffer (pH 7.00), containing 15 M KCl, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM of dithiothreitol (DTT) and centrifuged at 12,000 g for 25 min. The supernatant was filtered through cheesecloth and the filtrate was centrifuged at 134,000 g for 50 min. The collected supernatants were filtered again and the resultant filtrate was referred as cytosol. The total protein content was determined by the Lowry method (Lowry *et al*., 1951).

Miniaturized assay for glutathione-S-transferase activity (GST): GST activities were measured (Habig and Jakoby,

1981; Isgor *et al*., 2010) against the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), by monitoring the thioether formation at 340 nm ($\varepsilon = 0.0096\ \mu M^{-1}\ cm^{-1}$). The total GST activity was initially measured at pH 6.5 in 100 mM potassium phosphate buffer with 1.0 mM CDNB and 1.0 mM GSH, at 25°C (Geylan-Su *et al*., 2006). The assay then miniaturized for microplate applications (Isgor and Isgor, 2011a). Briefly, the total GST activity was measured in a 100 mM potassium phosphate buffer at pH 6.5 with 2.4 mM CDNB and 3.2 mM GSH. In the assay, the compounds to be tested or DMSO (vehicle control), CDNB and reduced GSH were transferred to the microplate in reaction buffer (100 mM potassium phosphate buffer at pH 6.5) and incubated at room temperature (22-25°C) for 5 min. Upon addition of enzyme source (bovine liver cytosol), the microplate transferred to spectrophotometer and GSH-CDNB conjugate formation was monitored as an increase in the absorbance at 340 nm for 240 sec. Initial rates of enzymatic reactions were determined as nanomoles of the conjugation product of GSH and reported as nmole/minute/mg protein. For the analysis of compounds, the kinetic results were expressed as inhibition of enzyme activity with respect to control (inhibition as % of control). The IC_{50} value was defined as the concentration of a compound required to achieve 50% inhibition of enzyme activity and determined with respect to the activity measured in the presence of vehicle alone (dimethyl sulfoxide). Since the kinetic inhibition profiles require the enzyme with 80% activity for screening purposes, here, the enzyme concentration that yields 80% of maximum signal in UV-VIS range was determined. Throughout this study, all the kinetic measurements for GST were performed at this concentration, unless otherwise stated, at 340 nm in 96 well microplate, using Spectramax M2e.

Assay for Src tyrosine kinase and cytosolic SFK activity indirect *in vitro* kinase (IVK) assay: The activity measurement were performed using ProFluor Src-family kinase assay protocol (Cai *et al*., 2001) with some modifications. The pure enzyme c-Src (Promega) or bovine liver cytosol, was used at concentrations that yield 20% of the maximum fluorescent signal. Briefly, the molecules were mixed with Src-family kinase R110 substrate (Rhodamine dye bound SFK specific peptide), in reaction buffer containing enzyme and control substrate (Lam *et al*., 1995; Li *et al*., 2008; Lou *et al*., 1996). The kinase reaction was initiated with the addition of 100 μM ATP. After incubating the plate at 25°C for 45 min, serine protease solution was added to each well and incubated for another 45 min at 25°C. The protease reaction was terminated with the addition of inhibitor, sodium orthovanadate (Na_3VO_4), then the fluorescence signal of the liberated R110-peptide was read at a wavelength of 525 nm (Ex 460 nm). The decrease in fluorescence of each well inversely relates to kinase activity of the enzyme within the wells and comparison performed with respect to control wells. To address the issue of false negatives resulting from protease inhibition by test compounds 7-amino-4-methylcoumarin (AMC) was used as a control substrate for protease activity. Since this peptide substrate contains no phosphorylation site, its cleavage is independent of kinase activity and the AMC fluorescent signal, at 460 nm (Ex 355 nm) is not expected to change with Src inhibitors. The decrease or increase of signal intensity reveals if the observed kinase inhibition (with R110-bound substrate) is false positive (so protease inhibitor) or not (Grant *et al*., 2002; Li *et al*., 2008). All measurements for indirect IVK assay were performed using Spectramax M2e.

Direct *in vitro* kinase assay by ELISA (ELISA-IVK) for Src tyrosine kinase, total tyrosine kinase and cytosolic tyrosine kinase activity: The inhibition of the synthesized compounds was verified by virtue of the ELISA based *in vitro* tyrosine kinase assay (Takara Universal Protein Tyrosine Kinase Assay, Tokyo, Japan), where the phosphorylation level of substrate peptide immobilized on plates was explored with Horse Radish Peroxidase (HRP) conjugated anti-phosphotyrosine (pY20) antibody. The substrate is synthetic peptide with poly (Glu, Tyr) sequence with wide spectrum of tyrosine kinase specificity. The concentrations of the enzyme used to construct the calibration curve were as follows: 195, 65, 21.7, 7.22, 2.41, 0.802×10^{-5}, units μL^{-1} for Src. The assay was initiated with the addition of ATP, in a final assay volume of 50 μL at 37°C. The change in absorbance at 450 nm was measured to determine the end-point of the kinase reaction. The inhibitory effect of compounds or drug, on tyrosine kinase activity was monitored by the diminished activity of kinase at 450 nm. The dose-response curves were constructed in the presence of varying doses of compounds and the IC_{50} values were determined as the concentration of a compound required to achieve 50% inhibition of tyrosine kinase activity with respect to the control wells. For kinetic inhibition profiles of drug, the enzyme concentration that represents the 80% of overall activity was used for screening.

RESULTS

The initial experiments were performed to evaluate the effect of doxazosin on cytosolic GSTs using bovine liver cytosol preparation as enzyme source. Here, the amount

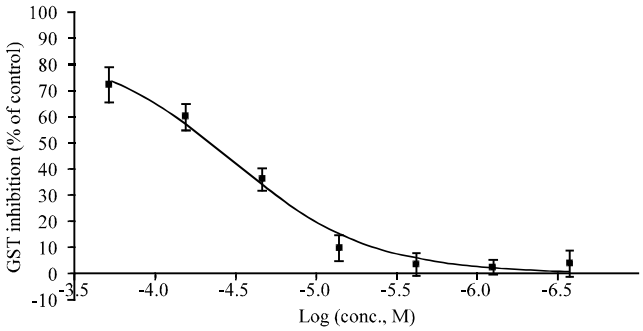

Fig. 2: The dose-response curve for cytosolic glutathione transferase against varying doses of doxazosin with IC_{50} of 38.419±4.182 μM

of cytosol that exerts 80% of maximum signal at 340 nm was determined as described under the materials and method section and found as 128 μg mL^{-1}. All the dose response studies with GST were conducted at this concentration and the results reported as % inhibition with respect to control, where the ethacrynic acid was positive and drug vehicle (PBS) was negative control. To improve the reproducibility of the assay, the drug was incubated for 10 min with the assay mixture including glutathione, CDNB and buffer. Each concentration of the drug was evaluated once in a set of varying doses. The duplicate of the experiment was performed, using the same enzyme source and drug doses, within 10 min following the kinetic measurement of the first concentration set. The dose response curve for doxazosin against cytosolic GST is given in Fig. 2 for 128 μg mL^{-1} of bovine liver cytosol, with IC_{50} of 38.42±4.18 μM for doxazosin. To determine the effect of doxazosin on tyrosine kinase activity, we designed two independent experiments both using recombinant Src tyrosine kinase and bovine liver cytosol as enzyme sources. In the first part, we investigated the possibility of the drug as a Src kinase inhibitor. For this purpose, we used commercially available, pure Src kinase in a fluorometric assay and constructed the calibration curve for pure enzyme (Promega Corp., USA) and determined that 155.82±8.43 $\times10^{-5}$ U μL^{-1} is the amount of enzyme that exerts 50% of the maximum signal and approximately 80% active enzyme was 249.3×10^{-5} U μL^{-1} (Fig. 3). The fluorescence signal for both peptide-R110 substrate ($\lambda_{Ex}/\lambda_{Em}$: 460/525 nm) and control probe AMC ($\lambda_{Ex}/\lambda_{Em}$: 355/460 nm). This activity level provides the most suitable linear range to determine the inhibitors in drug screening efforts. Then, at the same enzyme concentration, we analyzed the inhibitory activity of Doxazosin at varying doses (Fig. 4), where the IC_{50} was found as 10.93±1.34 μM. In the second part of the experiment, we investigated the possible inhibitory activity of the drug on bovine liver cytosol where the

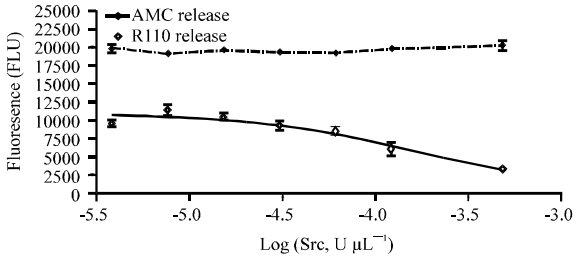

Fig. 3: Src kinase titration curve by IVK method resulted in the EC_{50} of 155.82×10^{-5} U μL^{-1}

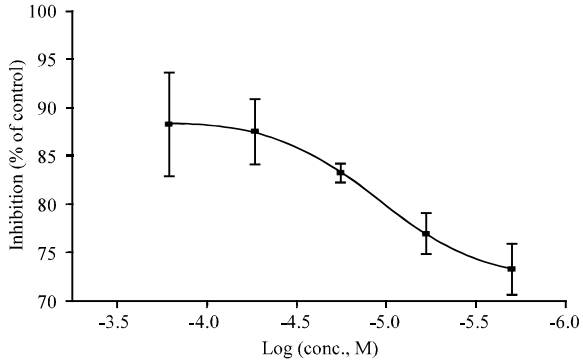

Fig. 4: Dose-response curve for varying doses of doxazosin as percent inhibition exerted on Src kinase, where the IC_{50} was 10.93±1.34 μM

soluble GSTs and protein tyrosine kinases highly abundant. The effect of drug on total tyrosine kinase activity was measured by using ELISA-IVK method with cytosol as enzyme source. Here, the cytosol that exerts 80% of total enzyme activity was determined as 1.457 μg mL^{-1} that was corresponding to 150×10^{-5} U μL^{-1} kinase activity with respect to tyrosine kinase titration curve (Fig. 5). The effect of doxazosin on total tyrosine kinase content of cytosol was determined with IC_{50} of 569.2±46.4 nM (Fig. 6). The inhibitory activity

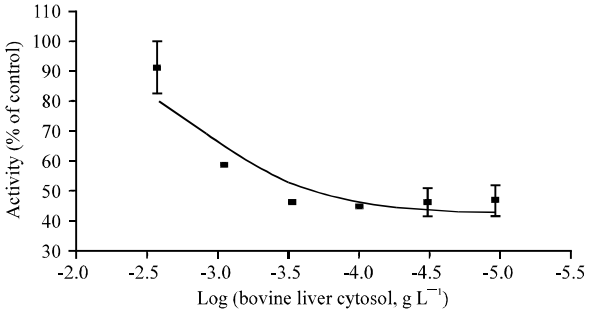

Fig. 5: The total cytosolic tyrosine kinase titration curve by ELISA-IVK resulted in the EC_{50} of 1.39±0.48 μg mL^{-1}

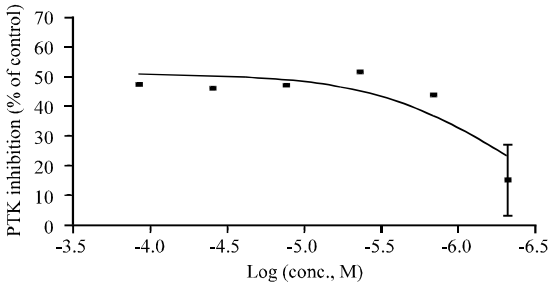

Fig. 6: Dose-response curve for doxazosin at 150×10^{-5} U μL^{-1} total PTK activity of cytosol was determined with IC_{50} of 569.2±46.4 nM

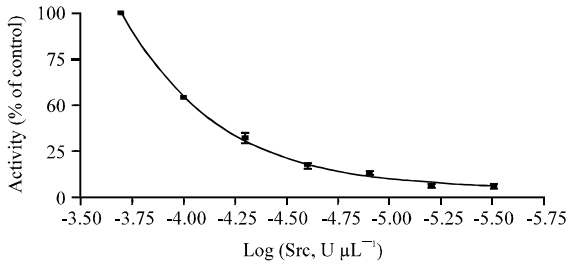

Fig. 7: Src kinase titration curve by ELISA-IVK resulted in the EC_{50} of 159.5±5.03×10^{-5} U μL^{-1}

of drug towards Src was evaluated using the same method, where the calibration curve with recombinant Src tyrosine kinase (Takara Inc., Japan) was constructed (Fig. 7). The 50% active enzyme was determined as 159.5±5.03×10^{-5} U μL^{-1} and 80% active enzyme was calculated as 251.2×10^{-5} U μL^{-1}. At this enzyme concentration, the IC_{50} value was determined as 186.9±20.2 nM (Fig. 8). Then, to analyze the total Src kinase activity of cytosol using IVK method with peptide-R110 substrate, we optimized conditions for the use of cytosol as enzyme source, since this method is suitable

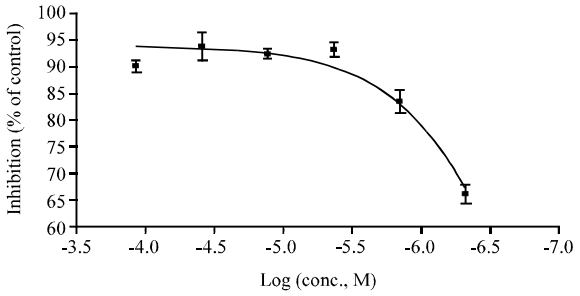

Fig. 8: Dose-response curve for doxazosin at 251.2×10^{-5} U μL^{-1} Src activity resulted in IC_{50} of 186.9±20.2 nM

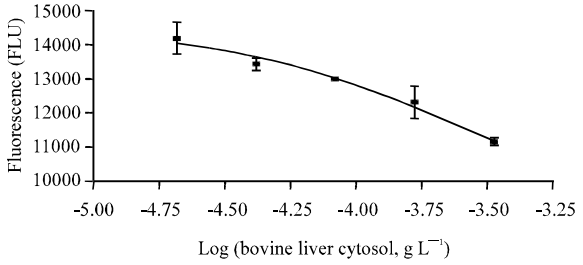

Fig. 9: Total cytosolic SFK titration curve by IVK resulted in the EC_{50} of 0.2337 μg mL^{-1} of cytosol

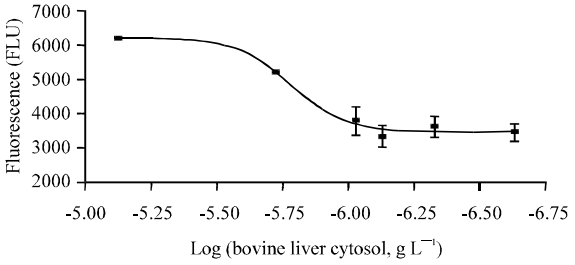

Fig. 10: Dose-response curve for Doxazosin at 0.3210 μg mL^{-1} of cytosol, representing 80% activity of total cytosolic SFK, resulted in IC_{50} of 1.643±0.461 μM

for High Throughput Screening (HTS) application and eventually optimized for the use of pure enzymes. Total SFK activity of cytosol was measured (Fig. 9) and 50% active total SFK was determined as 0.2337 μg mL^{-1}. The 20% signal was observed approximately at 0.3210 μg mL^{-1} of cytosol and at this concentration, the effect of doxazosin that exerts inhibition of total SFK activity was also evaluated. The dose-response curve for this assay (Fig. 10) resulted in IC_{50} of 1.643±0.461 μM.

DISCUSSION AND CONCLUSION

Several studies have invested efforts to clarify the mechanisms underlying doxazosin-induced apoptosis, including *in vitro* and *in vivo* studies with various tumor cells, endothelial cells and cardiomyocytes. In these studies, the main biological mechanism was established as the inhibition of receptor mediated signaling, based on the fact that quinazoline derivatives as tyrosine kinase inhibitors. Since most of the kinase activity inhibitors designed to interfere with ATP binding, the inhibitor molecule possess at least two aromatic rings. In this context, pyrazolo-[2,3-d]pyrimidines, pyrrolo-[2,3-d]pyrimidines, pyrido-[2,3-d]pyrimidines, amino-thiazoles, quinoline carbonitriles and quinazolines were proven successful ATP binding inhibitors (Schenone *et al*., 2011). Like other heterocyclic ATP analogs, quinazoline nucleus was shown to occupy the adenine region of the ATP binding site of the kinase. Hence, it is not surprising that doxazosin may exert apoptosis induction, independent of adrenoceptor-mediated mechanisms, by interfering directly with tyrosine kinase signaling with or without receptor mediation (Arencibia *et al*., 2005; Hui *et al*., 2008; Shaw *et al*., 2004). RTK associated signaling complexes employ Src or interact with, to transduce the signal and develop full biological response. The importance of Src inhibition on therapeutic effectiveness of the drugs which targets nuclear components, was also shown. Similarly, Dasatinib was shown to suppress the metastatic and invasive character of prostate cancer cells when combined with conventional chemotherapeutics (Lara *et al*., 2009). Doxazosin induced apoptosis in model cell lines was shown to employ the signaling components and associated with membrane bound receptor activation or involvement (Gan *et al*., 2008; Giardina *et al*., 2009; Keledjian *et al*., 2005; Kyprianou, 2003; Partin *et al*., 2003). None of these studies mentioned on any inhibitory activity of drug against tyrosine kinases and yet, there is no study tailored to search the effect of Doxazosin on SFKs or other nonreceptor tyrosine kinases so far. Our previous efforts on searching kinase inhibitors were brought the attention on drugs with limitations; especially resistance due to GST involved mechanisms (Griffith *et al*., 2010). The remarkable examples showed that the GST activity was modulated by the overactive kinases through phosphorylation of tyrosine, threonine or serine residues on GST (Okamura *et al*., 2009; Singh *et al*., 2010; Townsend *et al*., 2006). Similar to acquired resistance towards cytotoxic platinum drugs, the lessened effectiveness against tyrosine kinase targeting drugs was found as time and dose dependent and possibly linked to oxidative stress and GST over activation, as reviewed before (Isgor and Isgor, 2011b). While continuously investigating for new drug leads, the efforts in identifying the novel targets of well-known therapeutics appeared to be important to develop multifunctional drugs with improved benefits which approach was proven useful with examples of thalidomide and imatinib (Da Fonseca and Casamassimo, 2011). Hence, we focused on currently available drugs with fused heterocyclic rings and searched for possible tyrosine kinase inhibitors with structural resemblance to drugs already caused off-target toxicity on cells, with no known kinase inhibitory potential yet, such as Doxazosin. Although several tyrosine kinase inhibitors are in clinical development in patients with solid tumors, since 2007, there are only 19 clinical studies with Doxazosin regarding its effect on benign prostate hyperplasia, hypertension and urine retention. Neither preclinical, nor the currently available clinical studies mentioned on drug detoxification reactions through GST family of enzymes. The early study was only shown that doxazosin is extensively metabolized in the liver mainly by O-demethylation of the quinazoline nucleus or hydroxylation of the benzodioxan moiety (Kaye *et al*., 1986). However, glutathione transferase enzymes are also highly abundant in liver, where most of the drugs are subjected to GST mediated S-glutathionyl conjugation. Therefore, glutathione conjugation is extensively exercised way of drug solubilizing and detoxifying mechanism. Accordingly, we investigated the effect of doxazosin on cytosolic soluble GSTs and found that total GST activity was inhibited at 38.4 μM which was actually less than the concentration to induce cardiomyocyte apoptosis *in vitro* (Eiras *et al*., 2006). Although orally administered single dose of 8 mg was reported to reach 0.122 μM in circulation, the amount of drug accumulated in intracellular compartment was accepted as 1 μM (Benning and Kyprianou, 2002; Frick *et al*., 1986). With the available information in the literature, we determined that the GST inhibition of Doxazosin is in safe limits. Very recently cytosolic GST activation was reported to participate in the ligand-independent activation of EGFR and related to imbalance in cellular oxidative status in cancer cell (Hayes *et al*., 2005; Owuor and Kong, 2002; Uno *et al*., 2011). Then, the GST inhibitory dose of the drug also seems to function in negatively controlling the stress-related EGFR activation. Since quinazoline derivatives inhibit RTK signaling directly and through the inhibition of RTK downstream components indirectly, thus, Doxazosin induced GST inhibition may benefit for controlling the kinase activity of proliferating cancer cells. This was possibly one of

the unnoticed reasons of apoptosis induction observed in model cell lines (Giardina *et al*., 2009; Gonzalez-Juanatey *et al*., 2003; Kyprianou *et al*., 2009). Another critical point for evaluating Doxazosin-GST interaction is based on the fact that drug is known to induce apoptosis through DNA and other nuclear components but only in certain number of studies (Arencibia *et al*., 2005; Eiras *et al*., 2006). Moreover, our previous and on-going studies in searching for dual acting inhibitors against GST and Src activity is based on the fact that most of the acquired drug resistance is attributed to GST over activity and it is true for drugs capable of interacting DNA or other nuclear components (Isgor *et al*., 2011). This explains the possible involvement of GST in drug metabolism and similar to platinum drugs, the apoptosis induction through nuclear translocation may be limited (Townsend *et al*., 2006). The Doxazosin induced apoptosis in breast and prostate cancer cells was typically identified with similar mechanisms controlled by other quinazoline based tyrosine kinase inhibitors (Chang *et al*., 2008; Gan *et al*., 2008; Giardina *et al*., 2009; Hui *et al*., 2008; Kyprianou *et al*., 2009). Considering the drug development efforts for Doxazosin was based on alpha-1 adrenergic receptor as the biological target, therefore, these earlier studies are actually the assessment of Doxazosin off-targets in anticancer drug discovery. Furthermore, these studies which invested efforts to clarify the mechanisms underlying doxazosin-induced apoptosis, are indicating its possible contribution to Src mediated signaling cascades. In support of this, it was previously reported that the quinazoline core of α1-adrenoceptor inhibitors is responsible for apoptosis induction capacity of these molecules (Kyprianou and Benning, 2000). However, there is no study tailored to delineate the effect of Doxazosin on SFK member enzymes, even though several quinazoline derivatives were already patented SFK inhibitors. In addition, there is no report searching the direct effect of Doxazosin on Src so far and this may be due to it was not designed to inhibit SFKs at first place. In this context, we questioned if Doxazosin may also act through the Src related mechanisms and analyzed its effect through *in vitro* acellular assays with both recombinant enzyme and tissue homogenates as enzyme sources. We identified that Doxazosin inhibits Src kinase at IC_{50} of 10.93 μM (with Src family kinase specific substrate). Doxazosin was found approximately 60 fold effective on SFKs than Src itself, were the SFK activity was determined using the general tyrosine kinase substrate (IC_{50} of 186.9 nM) and Src kinase activity using the SFK specific substrate (IC_{50} of 10.93 μM). Considering the same enzyme activity was used for both of these assays, the inhibition of the kinase reaction towards general tyrosine kinase substrate may also represent the behavior of the overactive Src kinase which has high phosphorylation capacity with low substrate specificity (Guarino, 2010; Ingley, 2008; Isgor *et al*., 2011; Lou *et al*., 1996). Then, we analyzed the inhibitory activity of the drug on bovine liver cytosol where the soluble GSTs and protein tyrosine kinases highly abundant. Using tissue homogenate (cytosol) as enzyme source, we determined IC_{50} values for total SFK and tyrosine kinase targets as 1.643 μM and 569.2 nM, respectively. The amount of cytosol used in these assays was reflecting the 80% active Src and representing the active enzyme at physiological conditions. With this enzyme source, 50% SFK inhibitory activity was determined as 1.643 μM by *in vitro* kinase assay. This is actually closer to the previously reported intracellular concentration of Doxazosin (1 μM) achieved *in vivo* by therapeutic doses prescribed for hypertension (Benning and Kyprianou, 2002; Eiras *et al*., 2006; Frick *et al*., 1986). Although the high concentrations of doxazosin was suggested to induce apoptosis in disease models with earlier studies, the currently available studies report varying values for different kind of tumor cells. The patented kinase inhibitors, as therapeutics for solid tumors, are actually the non-cytotoxic agents that induce growth inhibition and apoptosis in a prolonged exposure (Liao *et al*., 2010; Mayer and Krop, 2010; Nam *et al*., 2005; Yang *et al*., 2010). Hence, for apoptosis induction on a short-term exposure, the high dose application seems to be required. Doxazosin may not be one of the best kinase inhibitors for therapeutic applications for now but our present work displays the reasoning for early observations of prostate cancer growth inhibition. Moreover, the observed low resistance upon prolonged therapy regimens with Doxazosin could be attributed to its inhibitory activity towards cytosolic GST enzymes. The shared signaling components between RTK, SFK and GST family enzymes are important in drug targeting for therapeutic intervention, such as cell viability, migration, differentiation and proliferation. In this concept, the efforts in identifying the novel targets of well-known therapeutics are important to develop multifunctional drugs with improved benefits. Their effects on the off-target cellular components are also important to evaluate their capability to establish therapeutic combinations to reduce drug resistance (Wu *et al*., 2011; Wyatt *et al*., 2011). Doxazosin, as a drug with detailed human trials and use, may suggest a suitable treatment

option with well-tolerated side effects, in combination with therapeutics properly designed for tumor targets.

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