THE EFFECT OF COMBINED DRUG TREATMENT ON HL60 LEUKEMIA CELL SURVIVAL : DRUG REPURPOSING

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Abstract- Drug discovery and development efforts generally do not provide a new drug into the market but deliver the scientific information to be used in the development of diagnostics and therapeutics. Generally drugs highly metabolized by detoxification system enzymes, such as Glutathione Transferases (GST), are of the limited use in clinics. In that sense the aim of the work is to evaluate the novel effect of Doxazosin, the drug with antihypertensive effect, in combination with anticancer drugs on HL60 cells. The study was also designed to evaluate the effect of combined drug use against the GST target. The results revealed that the treatment of cells with 5 nM SU6656 resulting in GST activation was reversed in the presence of Doxazosin with almost 50% inhibition of that enzyme. On the other hand Genistein alone resulting 57% GST inhibition was not significantly lowered in the presence of Doxazosin. Therefore it may be suggested that use of doxazosin with isoflavonoid or indolinone derivative drugs may enhance drug effectiveness.

Keywords- doxazosin, SU6656, Genistein, Glutathione Transferase, Cytotoxicity, combined therapy, drug discovery.

I. INTRODUCTION

Drug discovery and development is a tedious and expensive process with very low success rates. The process starts with designing a novel molecule with possible function against a target which is a biomolecule inside or on the surface of a cell, such as a protein, DNA, DNA related protein or any other biomolecule that has role in cell functioning [1].

In the field, the researchers of the pharmaceutical companies generally focused on a few biomolecules as drug targets and follow a few pathways to study interactions between drug and other possible targets. Most often the efforts do not provide a new drug into market but just scientific information useful for researchers. Considering its low success rates to provide a new drug into the pharmaceutical market, new trend in the field is based on to search for new targets for well-known drugs, so called repurposing of drugs [2].

Repurposing is the newest and most accomplished approach to selectively identify and develop new drugs where a molecule currently approved by the Food and Drug Administration (FDA) to treat one disease with its safety and pharmacological attributes, is searched for treating another disease. In that approach, Quinazoline derivative drugs may provide new insights in employing novel aims with novel drug targets.

Quinazoline derivatives are known for decades as small molecule tyrosine kinase inhibitors and especially one of the most effective inhibitor types for Src Family Tyrosine Kinases (SFKs). Among quinazoline derivatives, Doxazosin is alpha 1-adrenergic receptor antagonists in clinical use for the treatment of hypertension and benign prostate tumors, rather than inhibitor for SFKs [3]. Although Doxazosin is in clinical use for antihypertensive treatment and improve urination in men with benign prostatic tumors (BPH), its derivative, Tamsulosin, is at clinics for benign prostatic tumors (BPH). Moreover, its potential therapeutic benefits for several forms of cancers were shown with Doxazosin-induced cell viability studies, and only verified with growth inhibition and apoptosis results [4].

Another family of drugs are derivatives of isoflavonoids and they are commonly used in clinics for cancer treatment. Among these, Gefitinib is used for non-small cell lung cancer, Saracatinib is used for advanced prostate cancers and shown to decrease the metastasis rate. In addition to these, Genistein, an isoflavonoid, is found as inversely associated with the risk of cardiovascular diseases [5], an Antiangiogenic agent, an inhibitor of Protein Tyrosine Kinase (PTK), a competitive inhibitor of ATP in other kinase reactions, and also an anticancer therapeutic which is not limited to hormone dependent cancers [6]. However its clinical use is limited due to its unspecified behaviour against cancers types, such that drug was reported in some types a positive and in others with negative impact on disease progress.

Another drug developed but failed in clinical use due to its nonselective inhibitory action against various cellular components is SU6656. It is a small-molecule indolinone that effectively and inhibits Src family tyrosine kinases (SFKs). It was developed by the biotechnology company SUGEN Inc (a subsidiary of Pharmacia) in 2000 [7]. Potential drug candidate to limit tumor propagation of malignant B-cell lymphomas, but never reached to phase II clinical trials due to dosage limitations with high toxicity on trials. However, it is used for research efforts as an experimental inhibitor.

Indolinone, quinazoline and isoflavanoid member of drugs were not previously reported in combined cytotoxicity studies or against detoxification system members, especially Glutathione-S-transferase,

responsible for drug resistance and limited clinical use of drugs. GSTs is a group of enzymes that utilize glutathione in various biotransformation reactions, to convert some compounds such as therapeutic drugs, carcinogens, and molecules involved in oxidative stress, into more water soluble and excretable conhjugate forms [8]. In general, GST enzymes play an important role in detoxification of damaging chemicals such as environmental pollutants, carcinogens and medicines used in chemotherapy. For this reason in cancer treatment especially chemotherapy, GST overexpression in tumors is associated with the resistance to therapeutics. The aim of the work, therefore, is to evaluate the novel effect of Doxazosin, a quinazoline derivative with antihypertensive effect, in combination with anticancer drugs, derived from indolinone and isoflavanoids, on HL60 cells. the study was also designed to evaluate the effect of combined drug use against the GST target.

II. DETAILS EXPERIMENTAL

2.1. Materials and Procedures

All chemicals used were either molecular biology or analytical grade, Cell line HL60 (human promyelocytic leukemia cell, ATCC-CCL-240) were previously received as a gift from the Biology Department (Middle East Technical University, Ankara, Turkey) as frozen stocks. The tyrosine kinase inhibitors Genistein and SU6656 were purchased from Sigma-Aldrich, St. Louis, MO, USA. 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 [9]. 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.

The kinetic and end-point detection to measure the glutathione (GSH) conjugation and cytotoxicity 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 plots were constructed using 3-4 independent experiments, each in duplicates.

2.2. Treatment of Cells with Drugs and Cytotoxicity Assay

Cell line was cultured and maintained in complete medium (RPMI 1640), supplemented with L-glutamine (2 mM), 5% FBS, streptomycin (100 mg/mL), penicillin (100 U/mL), at 37°C in a humidified atmosphere at 37°C and 5% CO_2.

Doses of Drugs on the treatment of HL60 cell line separately (Table 1) Genistein used in combination with Doxazosin (Table 2-A) and SU6656 (Table 2-B) used in combination with Doxazosin was in the range of 0-7.50 □M range, for HL60 cell density of $1.5x10^6$ cells/mL at the treatment time. For Enzymatic assays, the cells were incubated with drugs at varying doses for 48 hours under culturing conditions, Then the cells were harvested at 900xg and homogenized in 50 mM phosphate buffer pH 7, containing 2 mM DTT,1 mM EDTA (pH 7), 0.5 % NP40 and 2 mM PMSF. Upon centrifugation at + 4 °C, 9000 x g for 30 minutes, the supernatants of cell homogenates were collected and aliquoted in 0.250 ml volumes and stored at -80 °C until they are used [10].

Cytotoxicity analyses were performed by XTT and viability by trypan blue dye exclusion methods. Each analysis was performed twice where each dose was analyzed in triplicates in 6 and 96 well plates for trypan blue and XTT analysis, respectively.

Doxazosin Mesylate, µM	Genistein, µM	SU6656, µM
7.5	-	-
3.75	3.75	3.75
1,875	1,875	1,875
0.625	0.625	0.625
0.312	0.312	0.312
0	0	0

Table1: Doses of Drugs used individually on the treatment of HL60 cell line cytotoxicity Analysis

A

Doxazosin Mesylate µM	Genistein µM
7.5	3.750
7.5	1.875
7.5	0.625
7.5	0.312
7.5	0

B

Doxazosin Mesylate µM	SU6656 µM
7.5	3.750
7.5	1.875
7.5	0.625
7.5	0.312
7.5	0

Table 2: A) Doses of Genistein, and B) SU6656 in combination with Doxazosin on the treatment of HL60 cell line cytotoxicity Analysis

Table 3: Doses of Drugs and the % Cell Death on the Treatment of HL60 Cell Line

	Cell Death Percentage		
Dose, mM	Doxazosin	Genistein	SU6656
7.500	64.92308	-	-
3.750	50.69231	60.39	96.58
1.875	30.76923	48.83	96.23
0.625	6.307692	48.37	89.35
0.312	1.538461	42.21	85.15
0.000	0.000	0.000	0.000

2.3. GST Activity

GST activities were measured against the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), by monitoring the thioether formation at 340 nm (ε = 0.0096 µM-1 cm-1). The total GST activity was initially measured at pH 6.5 in 100 mM potassium phosphate buffer with 2.4 mM CDNB and 3.2 mM GSH, at 25°C [11]-[13]. In the assay, the compounds to be tested or DMSO (vehicle control), CDNB and reduced GSH were transferred to the microplate in reaction buffer

and incubated at room temperature (22-25°C) for 5 min. Upon addition of enzyme source (HL60 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. For the analysis of compounds, the kinetic results were expressed as either enzyme activity or inhibition of enzyme activity with respect to control (inhibition as % of control).

III. RESULTS AND DISCUSSION

3.1. Cytotoxicity Analysis

After treating the HL60 cell lines with doxazosin (Table 1, column 1) and Genistein (Table 1, column 2), 50% cell death was measured at doses higher than 1.875□M, 0.312 □M of the drugs, respectively. For SU6656 (Table 1, column 3), almost 80% cell death was observed with 0.312 □M of the drug. For the combined analysis of the cytotoxicity and cell viability analysis, for Genistein-Doxazosin combination no significant change in cell density was observed with respect to Genistein alone treatment Fig.1.(a). On the other hand, SU6656-Doxazosin combination was reduced the cytotoxicity induced by SU6656 with increased doses of Doxazosin Fig.1. (b).

3.2. GST Activity :

The effect of Genistein, SU6656 and Doxazosin on HL60 cytosolic GST activity was measured (Fig.2.) or each drug separately and in combination with Doxazosin at representative doses of drugs depicted from previous cytotoxicity analysis (Fig.1. (a & b) , Table 3)

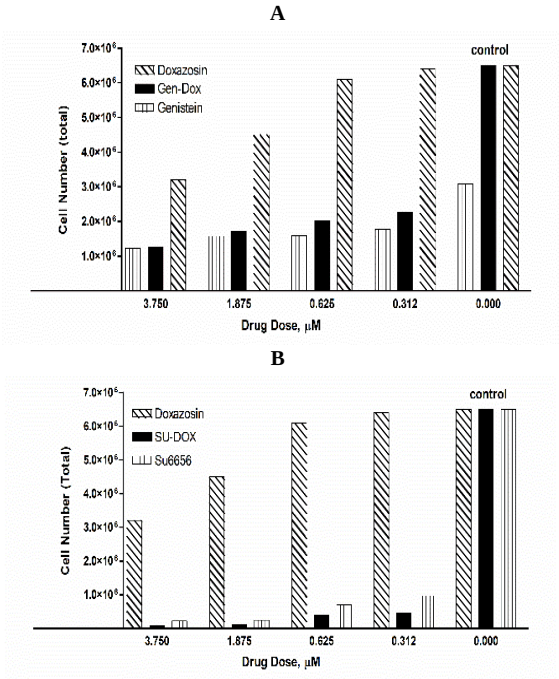

Fig.1. (a & b) Comparison of the effect of Genistein (A) and SU6656 (B) on cell toxicity separately and in combination with Doxazosin.

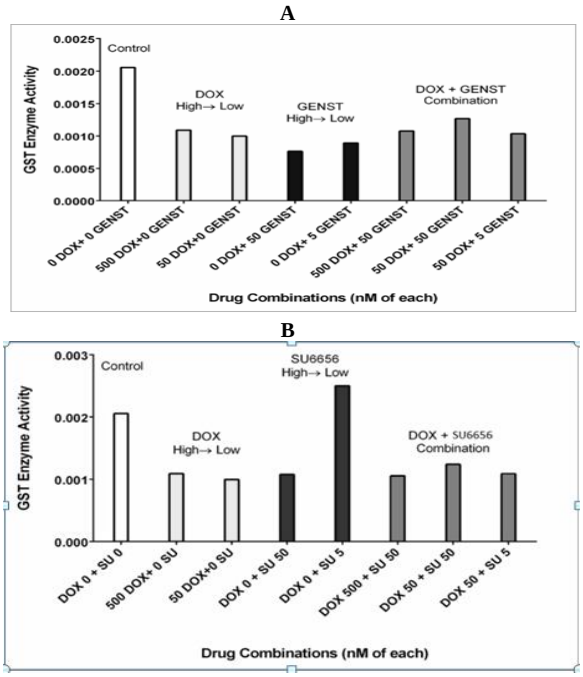

Fig.2. (a & b) GST Enzyme Activity of HL60 upon treatment with Genistein (A) or SU6656 (B) alone and in combination with Doxazosin. .

The change in enzyme activity upon treatment with drugs alone and in combination (Fig.2.) was also calculated as percent inhibition of the GST activity with respect to control (Table 4).

Table 4: Doses of Drugs and the % Cell Death on the Treatment of HL60 Cell Line

Drugs (nM of each)		% GST Inhibition
Doxazosin	SU 6656	
0	0	0
500	0	47.02389
50	0	51.54172
0	50	47.59186
0	5	-21.6*
500	50	48.692
50	50	39.70498
50	5	47.0556

CONCLUSIONS

In combination with DOX, SU6656 exhibited increased GST inhibition compared to inhibition observed when SU6656 was used alone in treatment.
In contrast, Genistein showed decreased GST inhibition compared to inhibition observed when Doxazosin Mesylate was used in combination.
At 625 nM, Doxazosin induced only 6%, Genistein and SU6656 induced 48 % and 89% cell death, respectively. It was decided that Doxazosin is not toxic to cells (less than 6% cell death) at 500 nM. So, the drugs were decided to be tested in combination with maximum 500 nM of Doxazosin. Since Genistein safe dose is 5 nM (viability results), when it is used alone to treat cells approximately 57% GST inhibition observed. In the presence of either 50 or

500 nM of Doxazosin, the inhibition with 5nM of Genistein was 48.6 and 47.1% respectively. Considering these inhibition values are not significantly different, the combined therapy wth Doxazosin and Genistein may be suggested especially for cancer patients having hypertension.

Low dosage (5 nM) of SU6656 appears to activate GST enzyme activity, however in the presence of 50 nM of DOX 47% inhibition of GST was observed. Since GST activity is related with Drug Resistance, the use of DOX with SU6656 derived drugs may be suggested to reduce such resistance at clinics.

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