CYP2D6 EXPRESSION IS CUMULATIVELY UP REGULATED IN MULTIDRUG TREATED HEPATOMA CELLS: A PREDECTIVE PHARMACOGENETICS IN VITRO MODEL

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ABSTRACT

Cancer and many other diseases require concomitant treatments with combinations of many drugs.Debrisoquine 4-Hydroxylayase (CYP2D6) is microsomal enzyme involved in phase I metabolism of a long list of drugs. Also, it is a marker of inter-individual variability in drug responsiveness. This study was designated to explore the regulation of CYP2D6 in hepatoma cells exposed to combinations of anticancer and epigenetic modifying drugs. HepG2 cell were treated with combinations of anticancer drug (Taxol), glucocorticoid (dexamethasone, DEX) and epigenetic modifiers: Trichostatin A (TSA) and 5 aza-deoxycytidine (5 aza-dC). The expression of CYP2D6 was determined by quantitative RT-PCR and compared to other CYPs and the corresponding cumulative apoptotic effect was determined by flow cytometry. The obtained results revealed thatUnder non-induced conditions, CYP2D6 was stably expressed and sub micromolar concentration of DEX mildly increased its expression. Individual treatments as DEX, Taxol, TSA and 5-azadC induced 2-6-fold increase in the transcript level, where the TSA was the most potent inducer. Combinations of 2 drugs as (Taxol+DEX), (Taxol+TSA), (Taxol+5-aza-dC) and (TSA+5-aza-dC). led to 3-10-fold increase (average 6.2), whereas cocktails of 3 drugs as (Taxol+DEX+5-aza-dC), (Taxol+DEX+TSA) and (Taxol+TSA+5-

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aza-dC) led to further up regulatory effect (11-27-fold). The highest increment (28-fold) was observed when cells were treated with 4 drugs as (Taxol+TSA+5-aza-dC+DEX). The progressive induction of CYP2D6 was correlated with the cumulative apoptotic effect (r=0.79). Conclusively, the data suggest, for the first time, that anticancer, epigenetic regulatory factors and dexamethasone cumulatively enhanced the baseline expression of CYP2D6.

Keywords: 5-Aza deoxycytidine, CYPs, dexamethasone, Taxol,

Trichostatin A

INTRODUCTION

Treatment of cancer and many other pathological disorders usually require concomitant supplementation with combinations of many drugs, which are differentially metabolized by a long list of Cytochromes P450 (CYPs). These CYPs are a family of ~60 members, widely distributed in different tissues and represent the main tool of phase I reactions involved in the biotransformation of drugs and other xenobiotics. The activity of CYPs is affected by their constitutive transcription factors, their genetic variability (polymorphism), and CYP-specific inducers or inhibitors. As liver is the main site of xenobiotics biotransformation, hepatic CYPs are influenced by modifiers including glucocorticoids(**Matoulková *et al.*, 2014),** epigenetic factors and many other xenobiotics(**Savas *et al.*, 1999**). One-third of CYPs are reported to be under epigenetic control(**Kacevska *et al.*, 2011).** In HepG2 cells, for example, inhibition of DNA methyltransferases (DNMTs) or histone deacetylase (HDACs) by 5 aza-2-deoxycytidine (5-aza-dC) and/or Trichostatin A (TSA) altered the expression of a large number of CYPs, suggesting the impact of epigenetic mechanisms on the regulation of CYPs(**Dannenberg and Edenberg, 2006)** . Also, CYPs play an important role in carcinogens and anti-cancer drug biotransformation. Among CYPs, Debrisoquine 4-Hydroxylayase (CYP2D6) has special importance, where it was introduced as a predictor of drug related responsiveness, inter-individual variation in addition to the relation between its polymorphism and the development of cancers and other pathological condition(**Bezerra *et al.*, 2018; Singh *et al.*, 2018).** These observations relay on some unique features of CYP2D6 including its high genetic polymorphism

with more than 130 allelic variants (databased in The Pharmacogenetic Variation **(Nofziger *et al.*, 2020)**, where it is the most investigated CYPs in relation to genetic polymorphism. Also, CYP2D6 protein has a wide activity range within a population that accounts the ultra-rapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs) and poor metabolizers (PMs) phenotypes**(Zhou, 2009).** This increases the usability of CYP2D6 in clinical pharmacogenetic testing**(Yang *et al.*, 2017).**

In liver, CYP2D6 is stably expressed and accounts for < 2% of the total hepatic CYPs and it mediates the metabolism in almost 25% of drugs**(Yu *et al.*, 2004; Zanger *et al.*, 2004; Zhuge *et al.*, 2004)**. HepG2 cells are predominantly used in the initial screening of drugs, the individual effects of many drugs and the involvement of different CYPs is well documented in the literature. Because CYP2D6 enzyme is responsible for the metabolism of antipsychotics, antidepressants, antiarrhythmics, and opioid analgesics**(Eichelbaum *et al.*, 2006),** the impact of anticancer drugs on its transcription was ignored in the literature. Moreover, studies that focus on the implication of CYP2D6 in multi-drug interactions are sparse. Thus, this work is designated to investigate the impact of concomitant multi-drug treatments on CYP2D6 expression in hepatoma cells compared to other CYPs members subjected to similar conditions.

MATERIAL AND METHOD

5-Aza deoxycytidine (5-aza-dC) was purchased from Sigma-Aldrich (CAS No. 2353-33-6<u>5)</u>., USA.Tricostatin A (TSA) was purchased fromSigma-Aldrich (CAS No. <u>58880-19-6)</u>., USA. Dexamethasone was purchased from Amriya for pharmaceutical industries Alexandria-Egypt and Taxol, produced by Ebewa pharma (Cat number A-4866) was purchased from the local market. In all experiments, a fixed sub micromolar (10 nM) of DEX was used. To induce histone deacetylation, cells were incubated with 5mM TSA, dissolved in dimethyl sulfoxide (DMSO) for 24 hrs.Hepatoma cells (HepG2) were generously provided by The Department of Cancer Biology, National Cancer Institute, Cairo University. Cell culture reagents Dulbecco's modified Eagle medium (DMEM), Fetal bovine serum (FBS) and penicillin, and streptomycin) were purchased from Lonza, BioWhittaker[®],USA.

Cell culture and treatment

Cells were seeded in DMEM with L glutamine, supplemented with 10% FBS, 1% Penicillin and Streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Initially, cells were cultured in a low cell density then passaged with defined cell densities according the experimental settings. Cells were cotreated with Taxol plus DEX and/or5-Aza-dC, TSA for 24**(Gagandeep *et al.*, 1999)**.Cells were cultured with initial cell density $3.5x10^5$cells/ml in 100 μlof media containing increasing doses of Taxol plus DEX and/or 5-Aza-dC, TSA for 24 hr.In addition to the control plates, the study included 11 different drug treatments. In 4 treatment modes cells were exposed to a single drug (Taxol, DEX, TSA or 5 aza-dC). Other treatments included cells cotreated with combinations of 2,3 or 4 drugs.

Apoptotic assay:

In order to determine the apoptotic effect, Annexin V FITC kit (MiltenyiBiotec, Auburn, CA, USA) was used following the plates by scraping and centrifuged at 1000 rpm for 5 min. The cell was resuspended in 1 ml PBS and then incubated with 0.25 μg/ml Annexin V reagent in 1X binding buffer for 15 min followed by two washes with Wash Buffer. Cells were resuspended in binding buffer containing 0.5 μg/ml propidium iodide (PI) then subjected to flow cytometry (BC Novus). The data was retrieved and analyzed by Kaluzasoftware**(Gagandeep *et al.*, 1999)**. All experiments were performed in triplicates where the average values were considered.

Expression analysis by real-time quantitative PCR:

The expression levels of CYP2C19 and CYP2D6 genes involved in drug-drug interaction were assessed using Qiagen Rotor-Gene Q-PCR Cycler 5 Plex. Total RNA was isolated using the GeneJET RNA purification kit, (ThermoFisher Scientific, USA) and 200 ng was used for cDNA synthesis using SensiFAST™ cDNA Synthesis Kit, (Bioline Inc, USA) following the manufacturer's protocol. 50 ng/μl (2μl) of cDNA was used as a template in cycling reactions mixture (20 μl) containing 50 nmol/μl (2 μl) of gene-specific primes,where the primers sequence of CYP2D6 sequence according to **(Steijns and Van Der Weide, 1998)** wereforward: 5′dATGAGAACCTGTGCATAG-3,reverse: 5′dCGGATGTAGGATCATGAG.3′ and GAPDH sequence according to**(Steijns and Van Der Weide, 1998)**were forward: 5-

CCTGCCAAGTATGATGACATCAAGA-3,reverse:5-
GTAGCCCAGG ATGCCCTTTAGT-3. For CYP2C19theforward:
5′dATTGAATGA AAACATCAGGATTG.3′ and the reverse:
5′dGAGGGTTGTTGATGT CCATC.3′(**Arefayene et al., 2003)**

The reaction mix included 10 *QIAGEN SYBR green*, raised to 20 μl
with ddH$_2$O then subjected a thermal cycling program consisted of a
single step of initial denaturation and 45 cycles (each consists of
single denaturation at 94 ^{O}C for 5 sec, annealing at 67 ^{O}C, 61 ^{O}C
(CYP2D6) and an extension step at 72 ^{O}C for 20 sec. Reactions were
terminated with a single step in which the temperature was increased
from 50 °C to 99 °C toensure the specificity of the amplicons and
produce a melt curve. In parallel, the expression of GAPDH was used
as an internal control to determine the fold expression changes in
target genes. The critical threshold (Ct) of target genes was
normalized with quantities (Ct) of GAPDH by using the$2^{-\Delta\Delta Ct}$ to
(**Livak and Schmittgen, 2001)**In qRT-PCR melt curves were justified
to assess whether the intercalating dyes have produced product
specific singles and to determine the Ct values.

Statistical data analysis

Apoptosis levels was measured and displayed as a percent of the
untreated cells and represented as the mean of 3 runs ± SD (standard
deviation). The expression data were presented as fold expression
relative to the GAPDH level. SPSS13.0 software package was used to
analyze the data and Microsoft excel was used in graphing. Principle
component analysis was performed with ClustVissoftwar.

RESULTS

1-Determination of cell apoptosis.

The apoptotic effects of Taxol, DEX, TSA and 5 aza-dC were
investigated by flow cytometry in which treated cells were dually
stained with Annexin V-FITC and Propidium iodide (PI). Figure 1
(panels A-C) demonstrates the scatter blots of flow cytometric
analysis, where the percentages of cells that underwent early and late
apoptotic fractions are represented in the lower and upper right
quadrates, respectively, whereas the percent of viable and dead cells
are shown in the lower and upper left quadrants, respectively. The

obtained measurements were compared to the obtained values of the untreated cells as a reference sample (Table 1). The results demonstrated that the apoptosis was drug dependent, where it was significantly induced in cells individually treated with Taxol (37.58%) or TSA (24%), whereas both DEX and 5-aza-dC induced negligibleapoptotic effects.   Treatment of cells with different drug combinations induced a variable apoptotic effect. This apoptotic effect was progressively increased along with increasing the number of drugs. The highest mean percentages of apoptosis were seen when cells were exposed to 3 or 4 drugs (35.8%, 40.9% respectively) (Figure 2). The fluctuation seen in figure 2 due to the variability of the apoptotic effect with different drugs and different combinations.

A

Taxol+Dex
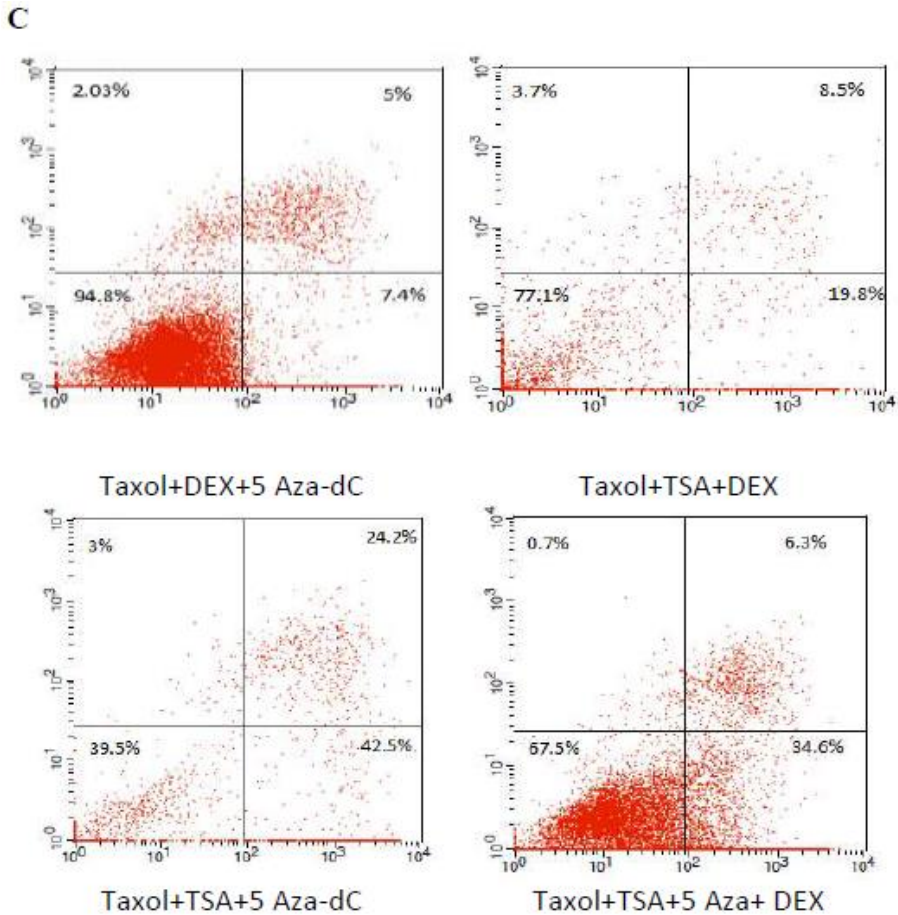
Taxol+TSA

Taxol+5Aza-dC

TSA+5 Aza-dC
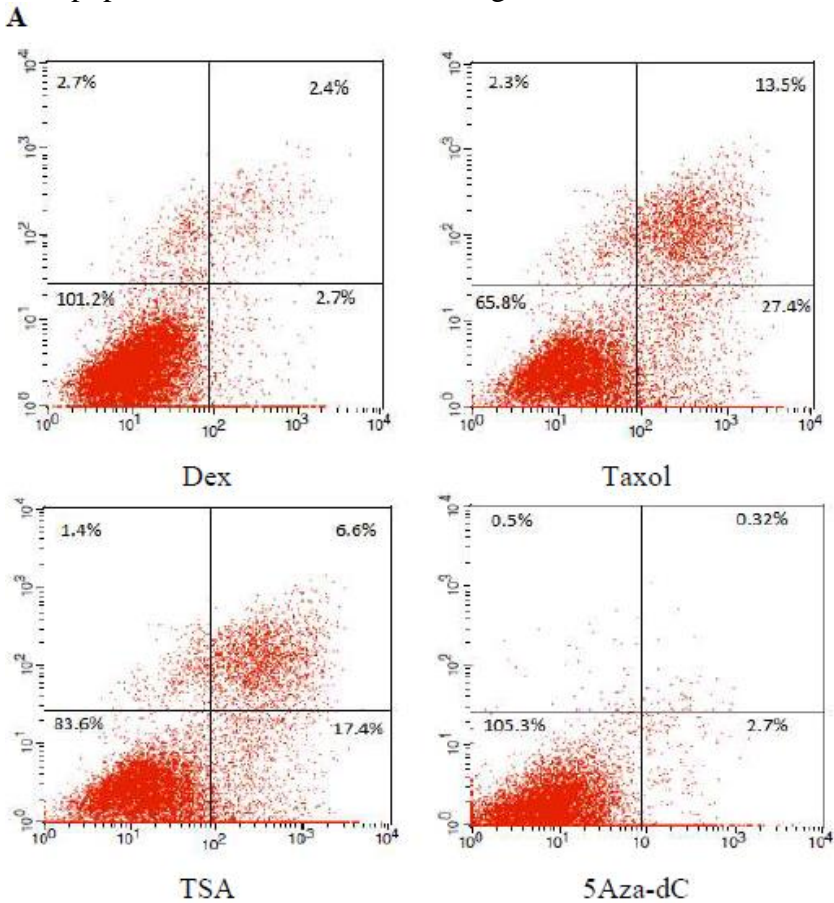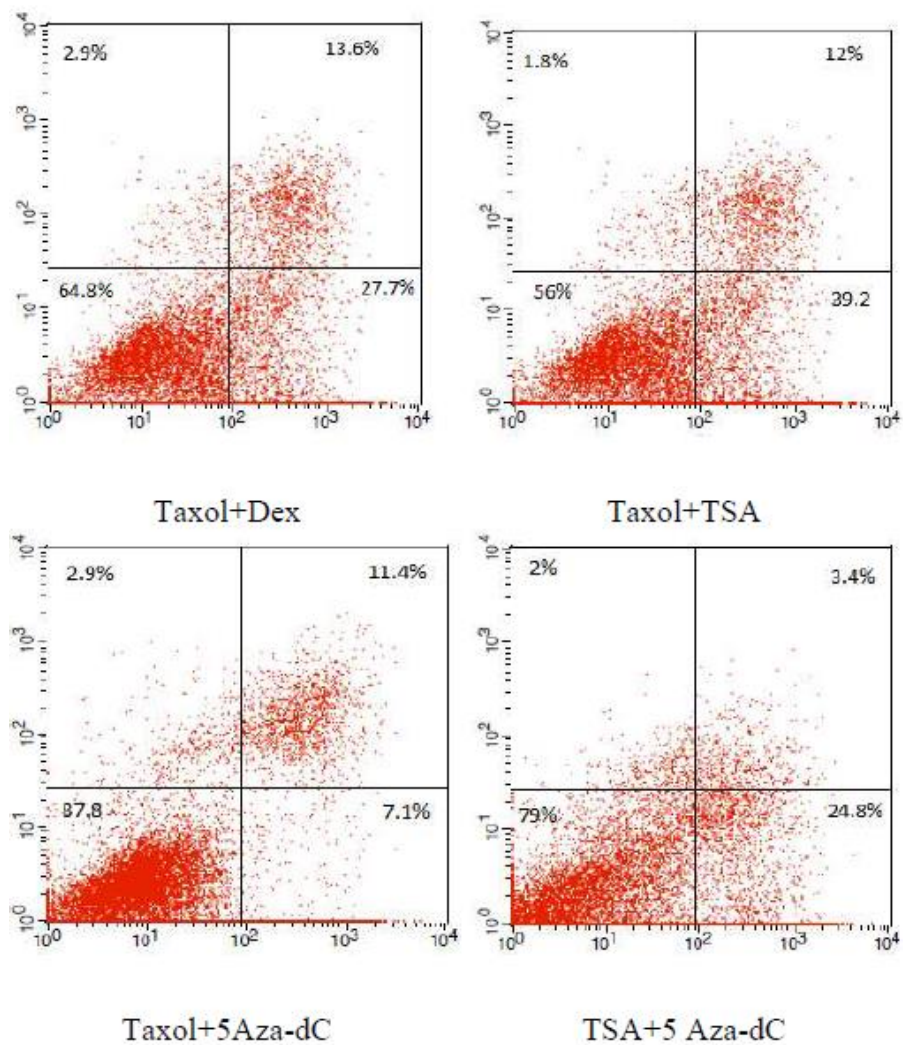
Figure 1. Apoptotic effect of different drugs combinations on HepG2 cells. Cells were treated with a single drug (A), combinations of 2 drugs (B), or combinations of 3 or 4 drugs (C). After incubation cells were stained with propidium iodide and subjected to flow cytometry to detect viable, early apoptotic, late apoptotic and dead cells shown in the lower left, lower right, upper right and upper left quadrants, respectively.

Abbreviations: Dex: dexamethasone, TSA: Trichostatin A, 5-aza-dC: 5-aza deoxycytidine.

Table 1: Percent of apoptotic cells and CYP2D6 relative quantifications in different treatments.

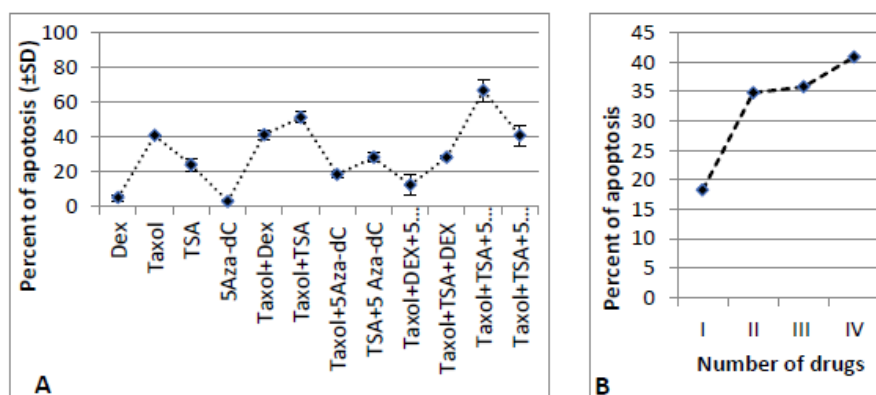| Treatment	Apoptosis (%)	RQs
Dex	5.1±1.76	1.41±0.20
Taxol	40.9±0.41	3.14±0.23
TSA	24±2.80	6.23±0.48
5Aza-dC	3±0.47	1.97±0.27
Taxol+Dex	41.3±2.50	4.56±0.48
Taxol+TSA	51.2±3.26	6.41±0.55
Taxol+5Aza-dC	18.5±2.03	2.94±0.65
TSA+5 Aza-dC	28.2±2.69	10.7±0.63
Taxol+DEX+5 Aza-D	12.4±2.98	11.47±0.49
Taxol+TSA+DEX	28.3±0.98	11.63±0.72
Taxol+TSA+5 Aza-dC	66.7±6.17	27.23±1.77
Taxol+TSA+5 Aza+ DEX	40.9±5.96	28.25±1.96

Figure 2. Variability of the apoptotic effects of combinations of Taxol, DEX, TSA and 5-aza-dC in HepG2 cells (A). In single treatments the apoptotic effect was drug dependent. Combinations of drugs induced a relative increase, where the highest percentage of the apoptotic cells is observed in cells exposed to cocktails of Taxol and/or TSA (D). Maximum apoptosis is seen in cells treated with 3 or 4 drugs (B).

2-Expression analysis

The expression analysis of CYP2D6 was determined in cells exposed to deferent single or multidrug treatments, where it was estimated as an expression fold relative to the GAPDH expression. Single treatments induced CYP2D6 expression following the order TSA>Taxol>5-az dC>DEX) (Figure 2, panel A). Combinations of 2 drugs led to 3-10-fold increase (average 6.2), whereas cocktails of 3 drugs induced more transcription (11-27-fold, mean 16.8). The highest increment (28-fold) was observed when cells were treated with 4 drugs (Figure 3, Table 1). Furthermore, the progressive induction of CYP2D6 was correlated with the corresponding cumulative apoptotic effect (r=0.79) (Figure 4). To investigate the mutual correlations between different combinations, principle component analysis was performed. Treatments with 2,3 and 4 drugs were clustered with limited distances, whereas cells population treated with 4 drugs did not pool with any of the other overlapping clusters.
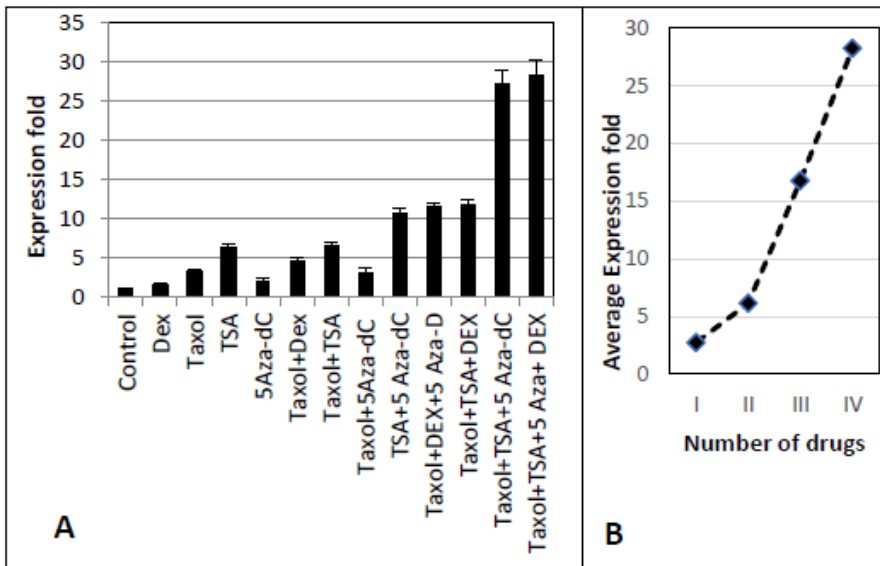

Figure 3. Changing pattern of CYP2D6 expression in hepatoma cells. (A) Cells were treated with different combinations of Taxol, DEX, TSA and 5-aza-dC (A). (B) The average increase of the gene transcripts when cells were treated with a single drug or concomitantly treated with 2, 3 or 4 drugs.
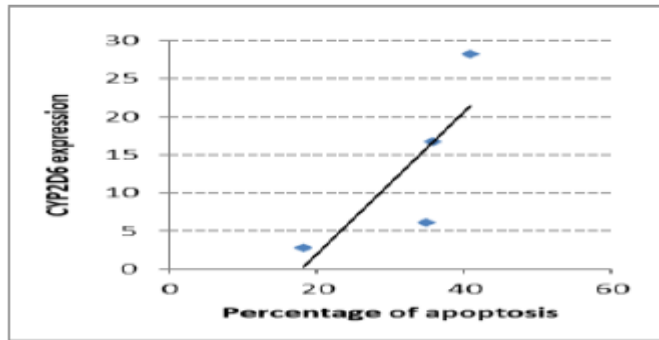
Figure 4: Correlation between apoptosis and CYP2D16 expression showing a cumulative apoptotic effect of Taxol, DEX, TSA and 5-aza-dC is correlated with their induction of CYP2D expression (Person correlation r=0.79).
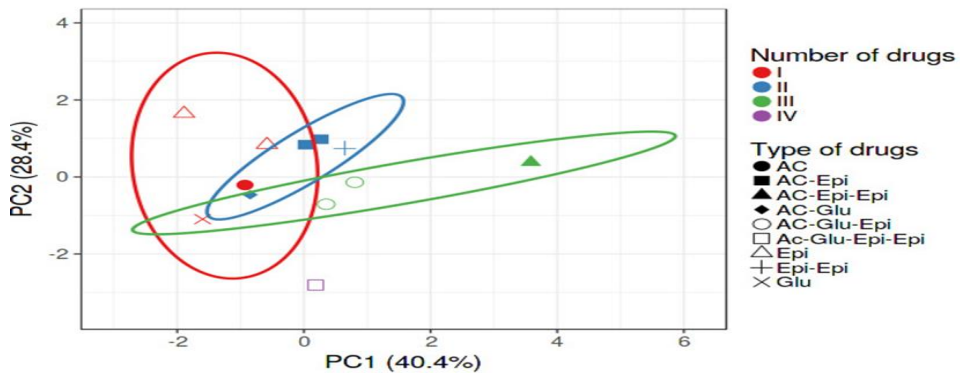

Figure 5:Principle components analysis of the expression biplot of CYP2D6 and 4 other CYPs genes (1A2,2B6,3A4,2C19) differentially expressed in HepG2 cells treated in 12 modes. X and Y axis show principal component 1 (PC1) and principal component 2 (PC2) that explain 40.4% and 28.4% of the total variance, respectively. Prediction ellipses demonstrates the clustering of cells treated with 1,2 or 3 drugs. Cells treatment with 4 drugs is represented by **hollow squire.**

DISCUSSION

The metabolic activity of CYP2D6 varies greatly between individuals, influencing dosing, efficacy and toxicity of CYP2D6-metabolized drugs**(Gaedigk *et al.*, 2008).**This reflects the wide range of its expression among individuals. Moreover, this gene is considered one of the most polymorphic CYPs. These characteristics qualified

CYP2D6 as a pharmacogenetics marker in some cancers (**Chan et al.,2020**). Herein, the study monitored the expression of CYP2D6 in hepatoma cells exposed to combinations of short list of drugs (2 to 4), including the anticancer drug (Taxol) predominantly used in treatment of many cancers, DEX, DNA methyltransferase inhibitor (5-aza-dC) and histone deacetylase inhibitor (TSA). This drug panel does not include any of the known CYP2D6 inhibitors. Also, no drug-drug interaction was reported among them. CYP2D6 was widely considered to be non-inducible on the basis of hepatocyte studies. However, other in vivo data suggested its induction by both endo-and xenobiotics (**Farooq** *et al.*, **2016**). The data presented here may challenge the previously reported studies that indicated the none or modest inducibility of CYP2D6 in most commonly used in vitro system of human hepatocytes(**Dixit** *et al.*, **2007**). The baseline expression of CYP2D6 mildly increased (1.4-fold) in cells treated with 10 nM DEX for 24 h, the observation was previously reported in HepaRG cells treated with higher concentrations of DEX for a longer period(**Pan** *et al.*, **2017**). The therapeutic use of DEX is well documented, where it reduced inflammation, used with other drugs to treat some types of cancers and cancer associated complications. In addition to DEX, the other drugs we used have a well-established anticancer effect (Taxol) or have potential anticancer role mediated through the epigenetic modulations such as TSA (**Chen** *et al.*, **2019**)and 5-aza-dC (**Tao** *et al.*, **2012**). Theyvariably induced 3, 6 and 2-fold increase in CYP2D6 transcription, respectively. Furthermore, co-treatments with different combinations (2-4 drugs) revealed progressive enhancement of the transcription. These data may present a newly developed observation, because before 2015 candidate anticancer drugs were not screened for putative CYP2D6 induction nor mandated by regulatory agencies including US Food and Drug Administration (FAD). Later, FDA included CYP2D6 for use in index clinical drug-druginteraction(DDI) studies (**Grimstein** *et al.*, **2019**). In human pluripotent stem cells-derived hepatocytes (hESC-Hep), the mRNA levels of CYP2D6 was much lower than in primary hepatocytes (hPH) due to the hypermethylation of CpG islands of CYPs, whereas the enhanced expression of CYPs in primary hepatocytes (hPH) was consistent hypomethylation of the CpG sites and permissive histone modifications. Accordingly, we observed that treatment with HDAC inhibitor (TSA) or DNMT inhibitor (5-aza-dC),

upregulated CYP2D6 by 6.3 and 1.97 folds, respectively. Combined treatment with both modifiers, further enhanced the transcript level to 10.7-fold. To validate this observation, the expression of a short list of other CYPs (CYP1A2, CYP3A4, CY2B6 and CYP2C19) was investigated under similar culture conditions. None of these genes showed cumulative response as CYP2D6. The inhibition of DNMTs and HDACs is translated into an inhibition of methylation and increase histone acetylation, respectively leading to the observed upregulation of their transcript. This may agree with previous reports in which co-inhibition of DNMTs and HDACs increased the transcription of CYP2D6 and other CYPs members **(Ru *et al.*, 2018)**.
 As CYP2D6is controlled bysmall heterodimer partner (SHP) and Krüppel-like factor 9 (KLF-9)**(Pan *et al.*, 2017),** as a transcriptional repressor and an activator, respectively, the epigenetic mediated upregulation of Klf-9 may explain the indirect effect on CYP2D6. Other scenarios may involve CYP2D6 copy number, especially populations studies have documented the high copy number variation (CNVs) of CYP2D6 relative to other CYPs**(Ramírez *et al.*, 2019).** Although CYP2D6 copy number in HepG2 cells was not identified, previous data showed that increasing the copy numbers in liver biopsy tended to increase up to 5 copies leading to the higher expression level of CYP2D6 but not for patients with more than 5 copies **(Wang *et al.*, 2011).** CYP2C19, which is closely related with CYP2D6 indrugtherapy of depression, gastroesophageal reflux and cardiovascular diseases, the gene did not show similar changing pattern to the CYP2D6. Surprisingly, comparative analysis demonstrated a good correlation (r=0.79) between the average apoptotic effects and the corresponding expression of CYP2D6.

  Although the expression data in this work is limited (60 data points: generated from 5 genes in 12 treatment modes), principle component analysis was employed to depict the overall behavior of cells under multidrug treatments. Cumulative data analysis demonstrated that cells treated with 4 drugs did not pool inside any of the prediction ellipses of cells treated with a single, 2 or 3 drugs. Furthermore, no observable clear distinctive clustering was found based on number of drugs as indicated by the overlapping ellipses areas.

Conclusively, the data presents HepG2 cells as an in vitro pharmacogenetics toll in which CYP2D6 expression is stably

expressed and progressively induced by anticancer (Taxol), glucocorticoids (DEX) and epigenetic modifiers (TSA and 5-aza-dC). More hepatic cell lines and in vivo studies are required to validate the implications for CYP2D6 in drug-drug interactions, drug metabolizing potential and screening of other anticancer and epigenetic modifiers.

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الملخص العربي

زيادة التعبير الجينى لجين انزيم CYP2D6 بشكل تراكمى فى خلايا الورم الكبدي المعالج بالادوية المتعددة: التموذج المختبرى للوراثة الدوائية التنبؤية

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يستلزم علاج السرطان وظروف باثولوجية أخرى التناول المتزامن لعدة عقاقير.ويعد إنزيم CYP2D6من الإنزيميات الميكروسومية الضرورية فى الطور الأول لأيض قائمة طويلة من العقاقير.كما يعد مؤشرللتباين بين الأفراد فى الإستجابة للعقاقير ومنبئ على قدرة الخلية على أيض العقاقير. تم تصميم هذه الدراسة لإستكشاف مدى تنظيم تعبير هذا الجين فى خلايا الكبد السرطانية بعد تعرضها لمجموعات علاجية مختلفة.ولبلوغ هذا الهدف تم معالجة خلايا الكبد السرطانية (HepG2) بعقارير تشمل العقار المضاد للسرطان (تاكسول) و الديكساميثاسون بالإضافة إلى مركبات معروفة بقدرتها على إحداث تحورات فوق وراثية تشمل الترايكوستاتين (TSA) و ٥-آزا ديوكسى سيتدين (5 aza-dC) حيث تم تقدير مستوى تعبير الجين بإستخدام تفاعل البلمرة المتسلسل الكمى ومقارته بنسب السيتوكروم ، كما تم قياس تأثير الموت المبرمج التراكمى للمركبات بإستخدام التدفق الخلوى. أظهرت النتائج أن الجين (CYP2D6) يتم ترجمته بصورة ثابته تحت الظروف الخالية من المؤثرات كما أن التركيزات الضئيلة من الدكساميثاسون زادت من معدل ترجمته بصورة ضئيلة، كما أظهرت المعالجات المنفردة الأخرى زيادة فى ترجمة الجين بمعدلات تراوحت من ٢-٦ ضعف ، حيث كان التاكسول و الترايكوستاتين الأكثر فعالية، وعند تعريض الخلايا للمجموعات علاجية مكونة من عقارين أدى إلى زيادة معدل ترجمة الجين بمعدلات تراوحت بين ٣ و ١٠ أضعاف (متوسط 6.2)، فى حين تزايد معدل الترجمة بمعالجة الخلايا بثلاث عقاقير مختلفة لمعدلات تراوحت بين ١١ و ٢٧ ضعف، ولوحظ أعلى معدل ترجمة للجين (٢٨ ضعف) بعد معالجة الخلايا بأربعة عقاقير.

وبصورة إستنتاجية تقدم هذه الدراسة إنزيم الـ CYP2D6 كمؤشر جيد على حساسية خلايا سرطان الكبد للعلاجات المتعددة كما تقترح النتائج أن عوامل المنظمة للتغيرات فوق الوراثية و مضادات السرطان و الديكساميثاسون (سواء كانت منفردة ومجتمعة) لها قدرات متباينة على زيادة ترجمة الجين.