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Anti-Neoplastic Role of the Triazole Analog TAN in Blocking Hedgehog Signaling Pathway Smoothened Receptors on the Human Colorectal Cancer Cell Line HCT116

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ABSTRACT
Developing a new effective anticancer agent is a real hope and challenge, in this concern protein specific targeting provides very selective and promising approach, on the other hand cell culture model enables the researcher to make a rapid surveying of a wide number of investigational agents. Hedgehog receptors (SMO) are of the key bio-targets that may ensure more effective anticancer mode of treatment as sonic hedgehog (Shh) is expressed in many cancer types. In this study, a prior computerized model of editing and surveying drugs was done. a candidate antineoplastic drug TAN was obtained for an in vitro hedgehog SMO receptors blocking effect assessment on HCT116, a human colorectal cancer (CRC) cell line in comparison with Itraconazole (ITC), and the standard anticancer agents doxorubicin (DOX) and 5- fluorouracil (5 FU) the known SMO blocking agent, together with the cytotoxic effect assessment on the HCT 116 cells and Vero cells for the above mentioned drugs. This research was designed to survey and assign a candidate drug with high affinity to block SMO receptors of Hedgehog signaling pathway of HCT 116 colorectal cancer cells. The results revealed that TAN may give a promising role in therapeutic approach of colorectal cancer. It showed an in silico parameters as anticancer agent by Van der Waals interaction of TAN force field with a scaffold like minor groove at twisted region of smoothed receptor. This interaction between TAN and this region was referred to as reliable mechanism of impairment of cell proliferation or invasiveness (according to In silico model software reliability). Also TAN has a significant antineoplastic effect on colorectal cancer HCT116 cell line represented by its significant decreasing of the cellular viability. In addition, TAN displayed significant safety characteristics from the measured parameters on Vero cell line in this research. We concluded that the SMO blocking investigational agent TAN has a reasonable and significant antineoplastic effect on colorectal cancer HCT116 cells represented by its significant decreasing of the cellular viability and reduction of target binding energy. TAN also displayed significant safety characteristics from the measured parameters on Vero cell line in this research.
Keywords: Renal Ischemia/Reperfusion Injury (RIRI), Sacubitrilate
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INTRODUCTION
Colorectal cancer is one of the leading causes of cancer-related fatalities worldwide [1-2], it is the 4th most common reason of cancer death after lung, stomach, and liver cancer [3]. CRC metastasis accounts for 40% to 50% of recently diagnosed cases, which is correlated with high morbidity [4]. Number of registered cancer cases was 5720, (31.05) per100, 000 in 1991 to 14,180 (44.46) per 100,000 population in 2008 in Iraq [5]. In spite of medical improvement in the management of CRC, the prognosis for cases with metastatic CRC is still poor. Little cancer stem cells (CSCs) are encountered in the CRC tissues, which is supposed to have the ability to metastasize and to result in resistance to anti-cancer drugs [6]. The duration, cost, and complexity of drug development make an early sorting; screening and selection of drug candidates occupy a priority score in diseases' therapeutic modalities progression [7]. In addition, one of the most precise approaches of assessing drugs against a given target is the rational target-based drug discovery, the surveying and assessment of the antineoplastic effects of candidate drugs in a human cell line of malignant cells is a reliable model from a Pharmaco dynamic point of view [8]. The surveying by the computerized model in combination with the in vitro model was performed in this research in order to test and evaluate the consistency of the outcomes achieved from these approaches. The highly predictive in vitro human cell culture model was preferred in experimental research to investigate mechanisms of disease as well as drugs' effectiveness and safety assessment and development. Where, the phenotype is similar to that in the human body [9]. Moreover, continuous cell line avoids many of the ethical aspects required in animal models [10]. Since, in medical research, good ethical principles disallow scientists from utilizing humans in studies that cause disease, danger and more than insignificant disagreeableness. Similar but not identical ethical guidelines were recommended in the work with animal models in vivo. So, the cell lines are extensively utilized in research as models of cancer and normal tissue [11].

MATERIALS AND METHODS
Cell lines
HCT-116 colorectal carcinoma cell line (ATCC® CCL-247™) USA
African green monkey kidney (Vero) cell line (ATCC® CCL-81™) USA

Smoothened protein solution (SMO)
This protein was purchased as containing a concentration of 130 μg/ml and for the applying of the assay of Docking and calculation of binding activity this

protein solution was prepared in a final concentration of 10µg/ml.

(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide MTT solution

This solution was prepared by dissolving 5mg of MTT powder in one mL of phosphate-buffered saline which is filtered and kept in the dark at 4°C [12].

Drug solutions for docking assay

TAN and ITC solutions

These solutions were prepared in a final concentration of 100 µg/ml for docking and 1000µg/ml for application of cytotoxicity assay, micro titration of these compounds was done as the experiment required.

Doxorubicin (DOX)

The molecular weight for this drug is 580.0 and chemical formula: C27 H29NO11, HCl. This solution was prepared in a final concentration of 1000 µg/ml for application of cytotoxicity assay, and for use micro titration of this compound was done as the experiment required.

5-Flourouracil (5-FU) solution

This drug was purchased as contains 500mg/10 ml, from which the solution was prepared in a final concentration of 1000 µg/ml for application of cytotoxicity assay, and for use micro titration of this compound was done as the experiment required.

Methods

Model of SMO-test drug

The computerized model of the target SMO is a critical step prior the test drug screening, so the model had been accomplished with Internal Coordinate Mechanics (ICM) package as accurate ligand binding mode and highly successful virtual screening with lower expense [13-14] by which the candidate anticancer drugs (TAN) have been edited and characterized within software. The target SMO protein ICM project was created and receptor and surface map was processed. The docking trajectory and docking energy of SMO-TAN binding was calculated and compared with the standard anticancer drugs, the higher energy of docking the higher anticancer effect. The drugs are, then, prepared in real laboratory steps for characterization and assessment of cytotoxic profiles.

ICM editor

It is a software extension for the ICM/pro-0/2013 molsoft for editing the candidate SMO target blocker with resultant outcomes that include general and kinetic properties of that test-blocker such as drug likeliness and molecular weight [15].

Docking assay application

TAN-SMO docking assay was performed by using a dialysis membrane and subsequent reading by UV spectrophotometer. This assay was applied by the using of dialysis membrane of 7000KD pore size (Sigma-Aldrich, TAN and other drugs were prepared in a concentration of 100 µg/ml, while the smoothened protein was prepared in a final concentration of 10 µg/ml.

Procedure

(1) Absorbance (ABS) measurement for untreated and undiluted glucose saline (GS).

(2) ABS measurement for TAN and ITC in constant concentration (100µg/ ml).

(3) Mixing of 0.5 ml of TAN+2.5ml of SMO in the dialysis membrane which has a pore MW of 7000 Dalton. This mixture is put in a beaker containing GS solution where this solution inside and outside the dialysis membrane should be equal in volume, the beaker is put on a magnetic stirrer for 30 min and then it was incubated at 37C for 1 hr.

(4) After incubation 1ml of the solution which presents outside the Dialysis membrane is pulled and retested for UV ABS and the difference in ABS before and after dialysis will be determined.

(5) Steps 2, 3 and 4 were repeated for each drug with ABS measurement and results recording.

(6) The solution inside the Dialysis membrane was kept in the refrigerator to be used in additional experiments.

Relative binding index

The binding index (BI) is calculated by dividing the drug concentration inside the membrane by the concentration of the same drug outside the membrane. The relative binding index (RBI) is calculated by dividing the binding index of 2 different drugs (software ICM model).

Drug cytotoxicity assay application

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is the method by which various compounds cytotoxicity can be assessed. The MTT colorimetric assay is a well-established procedure of detecting cell viability in proliferative, cytotoxic activity studies. This assay is built on the division of the yellow tetrazolium salt, MTT, to make a soluble blue formazan product by the enzymes of mitochondria (in cultured cells), & the amount of the produced formazan is directly proportional to the number of living, not dead cells, present during MTT exposure. Because the MTT assay is fast, suitable, & economical, it has become a very popular procedure for quantification of viable cells in culture [16]. Cell lines were seeded onto 96 well plates with a concentration of 1.0xl05 cells/ml. After incubation for 24-48 hr. at 37°C, when the confluent monolayer of HCT116 cells was complete (80% - 100%), different concentrations (1,10,20,40,60,80,100,500 and 1000µg/ml) of microtitered composites including TAN, 5FU, DOX, and ITC, were added to cultured wells at a final volume of 100 µl in each well except cells control in triplicate. After incubation at 37°C for 24 hr. in 5% CO2, the microtitered 96 wells plates were marched out & brought to biohazard safety cabinet by an aseptic environment to overcome any contamination, all used wells media were thrown away, The HCT116 cell monolayers were washed by PBS solution to remove any residual amount of composites or standard antitumor agents that may interact with MTT reagents,. Then all wells including drug treated cells, drug untreated cells & blank wells, were treated with 100 µl of maintenance media for each well, later, every well was supplied by MTT reagent (20 µl), after 4 hrs. of incubation at 37°C, 5% CO2, the formazan molecules were formed by a mitochondrial enzymatic process of the viable HCT116 cells, the dead cells were didn't form formazan particles because its mitochondria was disrupted. The formazan was dissolved by applying

diluted dimethyl sulfoxide DMSO (1:1) in isopropanol on each well including blank wells, by an ELISA reader , at 490 nm the absorbance was read with a reference wavelength of 630 nm, this procedure of MTT assay measurement was applied by many researches [17]. Mean blank absorption was subscribed from other samples and controls wells absorptions.

Dose Response curve and determination of IC50

The IC50 is the concentration required to inhibit the growth of 50% of the cell population and it can be determined by a dose-response curve. The change in the cell growth by different levels of doses was described by the Dose-Response Curve relationship after a certain time exposure; it is often referred to as a "graded" response because the estimated influence is continuous over several doses. The dose-response curve is essential to determine the safe and hazardous levels of the drug; in addition, it identifies the acute toxic effect of the drug and potential pollutants [18-20]. Dose-response curve was performed on drugs TAN, ITC, DOX, and 5-FU on HCT-116 cell line and VERO cell line since several doses of drugs were prepared in concentration (1,10,20,40,60,80,100,500 and 1000) µg/ml for investigational agents and for other chemicals, and this was accomplished by MTT assay.

IC50 determination

The IC50 of TAN, ITC, DOX and 5-FU was extrapolated from the dose-response graph. The drug concentration that reduced the viability of cells by 50% (IC50) was determined by plotting cell growth inhibition percentages versus a concentrations range [21].

The selectivity index (SI)

The selectivity index (SI) was determined by the ratio between IC50 of the investigational agents on the normal VERO cell line and IC50 of the same agents on cancerous cell line HCT116 [22]. The value of SI indicates selectivity of the agents to the cell lines tested. The agents that have SI value greater than two were considered to have a high selectivity towards cancerous cells [23]. Selectivity index (SI) = IC50 of the drug on normal VERO cell line divided by the IC50 of the drug on the cancerous HCT116 cell line.

Statistical analysis

Statistical descriptive measures (mean and median) and scattering measures (SD and SE) in addition to R^2 and ANOVA were done using IBM SPSS software for windows of version 21.0, the P value considered < 0.05 for all tests.

RESULTS

TAN-Smoothed Docking

Results of the computerized model of the investigational triazole derivative TAN -Smoothed docking interaction showed an obvious Van der Waals interaction of TAN force field with a scaffold like of amino acids residues. Another consideration was that TAN showed obvious drug likeliness, residues likeliness and had a scaffold likeliness mode of inhibition of protein blocking (according to docking model log binding results file), (Figures 1: A and B).

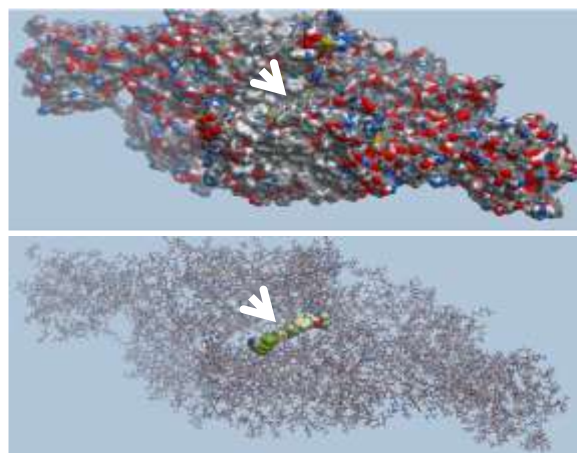

Figure (1- A): The virtual model of smoothened protein in Space-filling model (CPK model, calotte model) and ball and sticks model of ITC binding in its pocket site of the smoothened receptor obtained with ICM.

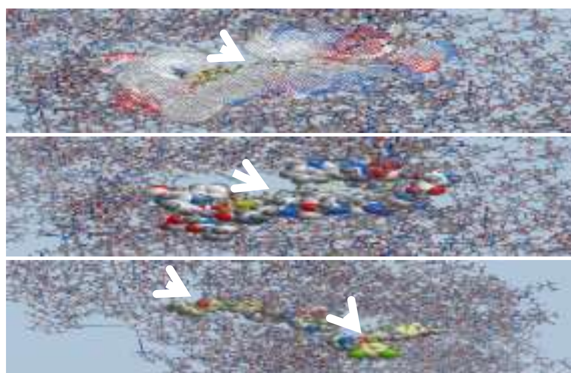

Figure (1-B): The virtual model of smoothened protein in the electron density map (EDM) model, ball and sticks model of TAN and Comparison of ITC and the investigational triazole derivative TAN, and The arrows refers to the binding site of smoothened receptor pocket, this interaction occurred at minor groove by Wan der Waals force .as revealed with ICM platform.

Table (1): The computerized model analysis the Types, energies and the binding residues of the test TAN in relation to the standards test Itraconazole. The total energy in kJ/m was determined for each compound using Gem dock version 2.

Table (1) had been obtained by applying the computerized model analysis showed specific types of energies that test drug (TAN) and ITC that bind to the target residues in number and type. Total van der Waals (VDW), hydrogen bond (H-bond) that enables to compare between test drug (TAN) and standard drug (ITC).

Table (2): Results of In silico model protocol showing energy terms presented by mean and RMSD (root mean square deviation) values

Map statistics (units are KJ/ mole)					
Term of positivity		Energy	VDW	H bond	Min Units KJ/mole
SMO-ITC	H-M ILE156	-121.38	-112	-8.64	-3.5
	H-S ASP165				-5.1
SMO-TAN	H-M ASN493	-118	-100.22	-15.58	-3.5
	H-S ASN 493				-3.5
	H-M ILE 496				-3.5
	H-S SER 1126				-5.1

Our results made investigational triazole derivative TAN a rational candidate for ongoing in vitro assessments on colorectal cell line model. This investigational triazole derivative TAN showed a considerable interaction with different regions (as scanned by the in silico model). However, little is known about the rule of Itraconazole as a sole agent or conjugated as anticancer agent, hence, our study can be regarded from first studies that pave the way for the use of investigational triazole derivative TAN as anticancer, and give future as alternative CRC therapeutic agent. High binding affinity of the ligand to the receptor is obtained by our docking simulations results. The value of R^2 (Pearson Correlation Coefficient) was 0.5754 as shown in figure 3, and this was regarded as positive correlation. Simulations supported the hypothesis that states the investigational triazole

derivative TAN is a potential ligand to target/ inhibit cancer cell. Results of in silico study will also guide the design of selective inhibitors of smoothed receptor with high specificity and potent activity in order to strengthen the therapeutic line available today against the dangerous colorectal cancer.

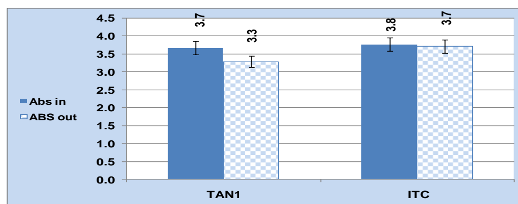

Figure (2): Docking status of TAN as compared with ITC; revealing the ABS differences between IN and OUT the dialysis membrane

There was obvious difference between in and out TAN in comparison with ITC which indicates that TAN had a more binding energy and hence a blocking effect to SMO even than ITC.

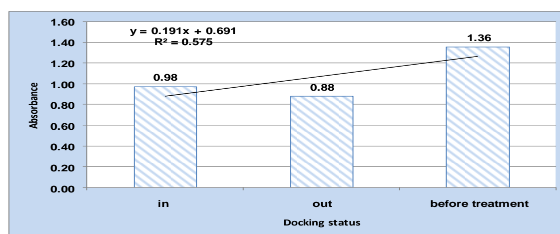

Figure (3): Docking status of TAN in and out of dialysis membrane which confirms the consistency of the higher affinity of the investigational TAN to block SMO

Calculation of Relative binding index

The binding index (BI) is calculated by dividing the drug concentration inside the membrane by the concentration of the same drug outside the membrane. The relative binding index (RBI) is calculated by dividing the binding index of 2 different drugs (software ICM model). Binding index (BI) = drug in / drug out; TAN BI = 3.7 / 3.3 = 1.12; ITC BI= 3.8 /3.7 = 1.02 RBI of (TAN, ITC) = 1.12 /1.02 = 1.1 This means that TAN was 1.1 more potent in binding to SMO than ITC although this difference was statistically not significant at $P < 0.05$.

MTT cytotoxicity assay and IC^{50} determination

The cytotoxic effect of TAN on HCT116 and Vero cells was assessed by MTT assay. A wide range of concentrations represented by (0, 1, 10,100,500 and 1000 µg/ ml) were used for the tested TAN and other drugs used in this study including: ITC and the standard anticancer agents (5-FU and DOX). After 24 hours incubation the Absorbance was measured for each well by ELISA reader and the percentages of growth inhibition (GI %) was determined. Figures (4) reveal the GI% for drugs on Vero cells while figure (5) shows GI% of drugs on HCT 116 cells. By using GI % for different drug concentrations we estimate the IC50, regression correlation coefficient (R^2) by plotting GI% against log of conc, for each drug. Table (5) shows the IC50, level of significance and R^2 for each drug on Vero cells, meanwhile table (6) shows the IC50, level of significance, R^2 and SI for each drug on HCT116 cells. SI was estimated by dividing (IC50 of drug in VERO cells / IC50 of same drug in HCT116 cells). Results TAN as compared with ITC effect on Vero and HCT116 cells presented by mean ± SEM were shown in tables 3 and 4, while values of GI% of TAN on VERO or HCT116 cells were shown in figures from 6 and 7 respectively.

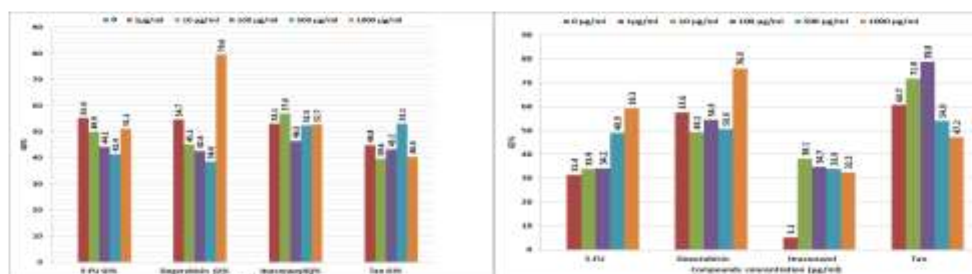

Figure (4): on the left shows the different GI% for drugs on Vero cells; while Figure (5) 0n the right shows the different GI% for drugs on HCT116 cells

Table (3): TAN effect on Vero Cell and HCT116 cells, presented by Mean± SEM of absorbance read at 490nm

TAN	N	Effect on VERO cells	GI% on VERO cells	Effect on and HCT116 cells	GI% on HCT116 cells
		Mean± SEM		Mean± SEM	
0	3	.10300±.051507	0	.11800±.011504	0
1	3	.13800±.002517	44.8	.03033±.009262	60.7
10	3	.14667±.011407	39.6	.03333±.005239	71.8
100	3	.14067±.007333	43.2	.02500±.015524	78.8
500	3	.12433±.003930	53.1	.05433±.016180	54.0
1000	3	.14533±.031897	40.4	.06233±.005207	47.2

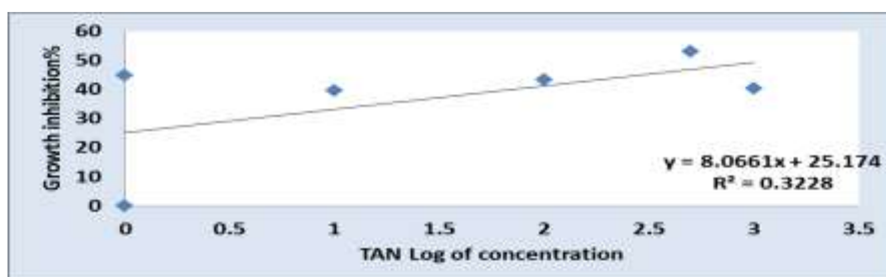

Figure (6): Cytotoxic effect of TAN on VERO cells presented by plotting of conce.log vs. GI% values

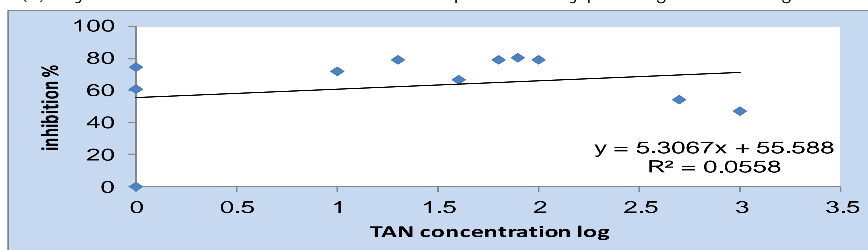

Figure (7): Cytotoxic effect of TAN on HCT116 cells presented by plotting of conce.log vs. GI% values

Table (4): Effect of ITC on Vero Cell and HCT116 cells, presented by Mean± SEM of absorbance read at 490nm.

ITC	N	Effect on VERO cells	GI% on VERO cells	Effect on and HCT116 cells	GI% on HCT116 cells
		Mean± SEM		Mean± SEM	
0	3	.10300±.051507	0	.11800±.011504	0
1	3	.13333±.004055	53.1	.13400±.011358	5.1
10	3	.12700±.003215	57.0	.09500±.017059	38.1
100	3	.14433±.007535	46.5	.09900±.002517	34.7
500	3	.13433±.007753	52.5	.10000±.005568	33.9
1000	3	.13400±.013577	52.7	.10167±.006173	32.5

Table (5): The IC50 and regression correlation coefficient (R) for each drug on Vero cells

Vero cells			
Drug	IC50	Level of Sig. of IC50 dif.	R
5 FU	601.434	0.205	$R^2 = 15.37$
TAN	428.062	0.454	$R^2 = 0.3228$
ITC	282.724	0.366	$R^2 = 0.243$
DOX	748.169	0.177	$R^2 = 0.3134$

Table (6): The IC50 and regression correlation coefficient (R) and SI for each drug on HCT116 cells

HCT 116 cells				
Drug	IC50	Level of Sig. of IC50 dif.	R	SI
5 FU	82.77	0.000	$R^2 = 0.5432$	7.27
TAN	63.94	0.001	$R^2 = 0.88$	6.69
ITC	21.85	0.004	$R^2 = 0.6906$	12.93
DOX	345.176	0.000	$R^2 = 0.2207$	2.16

The half maximal inhibitory concentration (IC50 which equal to 428.062 Mg/ ml) of TAN on Vero cells shows no significant difference with control, P value > 0.05 while it (IC50:63.94 Mg/ml) shows a concentration dependent (positive R since it is more than 0.5) highly significant inhibition difference on HCT116 cells as compared with control, P value was<0.05(P value :0.001). All other drugs including ITC, DOX and 5-FU show no significant difference in IC50 on Vero cells as compared with control ,although there was a variation among these drugs in the level of significance (DOX show least level: 0.17) but it was statistically no significant. All drugs have negative R^2 on Vero cells. On HCT116 cells, drugs other than TAN show highly significant difference from control in IC50 with the highest readings shown for 5-FU and DOX (P value: 0.000). R^2 was positive for all drugs except DOX.

Selectivity index (SI)

SI was estimated by dividing (IC50 of drug in VERO cells / IC50 of same drug in HCT116 cells) [22], table (6) and figure (8).

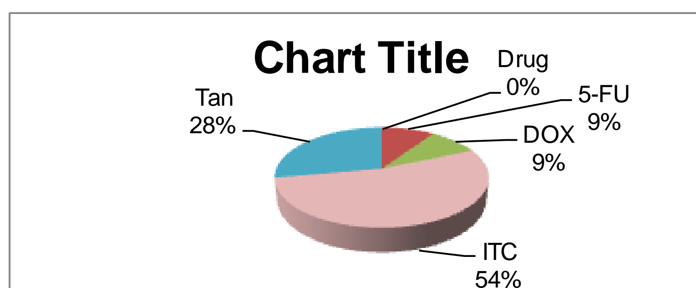

Figure (8): SI variations among drugs used in this study

The highest SI was observed with ITC (44%), 5-FU came in the 2nd place (24%) and TAN was the 3rd one (23%), (Samples with a SI greater than 2 were considered to have a high selectivity towards cancerous cells).As shown in figure 9 (A&B), TAN showed anticancer effect on HCT116 cell line while being safe to normal cells (VERO).

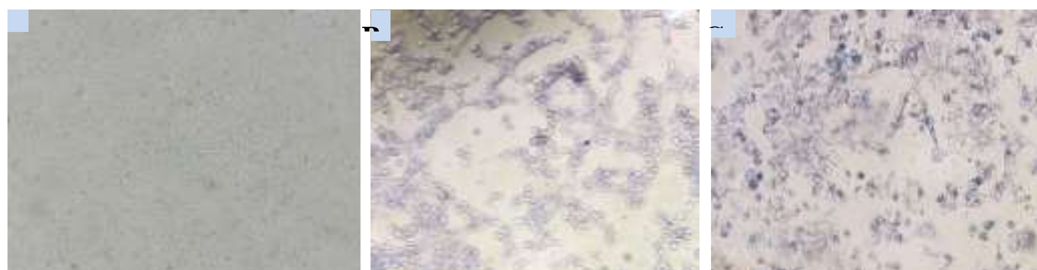

Figure (9)-A: A- HCT116 cell Line before treatment with TAN or addition of MTT reagent; B- HCT116 cell Line monolayer after **treatment with 63µg/ml of TAN; C: HCT116 cell Line monolayer after treatment with 100 µg/ml of** TAN.

DISCUSSION

TAN –SMO docking assay

In general TAN has shown a promising role in therapeutic approach of colorectal cancer. It showed an in silico parameters as anticancer agent by Van der Waals interaction of TAN force field with a scaffold like minor groove at twisted region of smoothed receptor (Van der Waals interaction is a distance-dependent interaction between atoms or molecules. Unlike ionic or covalent bonds, these attractions do not result from a chemical electronic bond). This interaction between TAN and this region was referred to as reliable mechanism of impairment of cell proliferation or invasiveness (according to in silico model software reliability). Our results made the investigational triazole derivative TAN a rational candidate for ongoing in vitro assessments on colorectal cell line model. This investigational triazole derivative showed a considerable interaction with different regions (as scanned by in silico model). However, little is known about the role of Itraconazole as a sole agent or conjugated in cancer therapy, hence, our study can be regarded as one of the first studies that pave the way for the use of the investigational triazole derivative TAN as anticancer, and give future an alternative CRC therapeutic agent. High binding affinity of the ligand to the receptor is obtained by our docking simulations results The value of R2, the coefficient of determination (Pearson Correlation Coefficient) was 0.5754, and this was regarded as positive correlation with RBI = 1.1 which means that TAN has one fold greater affinity to bind SMO than ITC . Simulations supported the hypothesis that states the investigational triazole derivative TAN is a potential ligand to target/ inhibit cancer cell. Results of in silico study will also guide the design of selective inhibitors of smoothed receptor with high specificity and potent activity in order to strengthen the therapeutic line available today against the dangerous colorectal cancer. Lucile Hoch et al in a preclinical trial

found that ITC binds to SMO receptors with high potency providing the first direct evidence that this molecule is a potent SMO antagonist [24]. Kim et al confirmed that ITC binds SMO at a site distinct from that for cyclopamine [25]. In the same context Li et al assure that ITC produce its anticancer effect by blocking the Hh pathway by preventing the accumulation of SMO receptor & preventing GLI release (a transcription factor) [26].

MTT assay

The half maximal inhibitory concentration (IC50) of TAN on Vero cells which equals to 428.062 Mg/ml, shows no significant difference with control, P value > 0.05 while it (IC50:63.94 Mg/ ml) shows a concentration dependent (positive R since it is more than 0.5) highly significant inhibition difference on HCT116 cells as compared with control, P value was<0.05(P value: 0.001). This reflects the high safety margin of TAN on normal cells and high efficacious effect on malignant cells. All other drugs including ITC, DOX and 5FU show no significant difference in IC50 on Vero cells as compared with control ,although there was a variation among these drugs in the level of significance (DOX show least levels of P value : 0.17) but it was statistically no significant. All drugs have negative R on Vero cells. Francis and Nayak had observed the cytotoxic effect of DOX on Vero cells; this result was also achieved by Fraczkowska et al [27-28]. Regarding SFU Al-Shammari, A et al documented the no significant cytotoxic effect of 5FU on Vero cells [29]. On HCT116 cells, drugs other than TAN show highly significant difference from control in IC50 with the highest readings shown for 5FU and DOX (P value: 0.000). R was positive for all drugs except DOX. The dose dependent cytotoxicity profile of 5FU on AMN3 cancer cells had been confirmed by Al-Shammari et al [29]. Fraczkowska et al, mentioned the significant cytotoxic effect of doxorubicin on leukemic cells [28] .Regarding the cytotoxic effect of ITC on cancer cells Kim et al, found that ITC was ten folds more potent than ketoconazole can suppress Hh pathway activity and the growth of medulloblastoma in a mouse allograft model and does so at serum levels.

SI

TAN shows the 3rd higher SI after ITC and 5FU (12.93 (44%), 7.27 (24%) and 6.69 (23%) respectively), so it has a good safety margin in comparison with DOX (2.16 (7%). Aftab et al found that ITC has potent and selective inhibitory activity on the proliferation of endothelial cells and multiple key aspects of tumor angiogenesis in vitro and in vivo in non-small cell lung cancer [30], whereas, Flis and Spławiński found that the SI of 5FU on Colo - 205, SW48 and HT29 CRC cells was: 0.4, 0.5 and 0.7 respectively, this indicates that 5FU has a highly significant selectivity index on HCT116 CRC cell than the above mentioned CRC cells. While the SI of DOX was confirmed by Prasetyaningrum, et al [31] as it was< 2.94 on MCF7 cells (in our study it was 2.16).

CONCLUSIONS

The SMO blocking investigational agent TAN has reasonable and significant antineoplastic effect on colorectal cancer HCT116 cell line represented by its significant decrease of the cellular viability (via MTT assay) and reduction of target binding energy(through ligand –protein docking assay). In addition, TAN displayed significant safety characteristics from the measured parameters on Vero cell line in this research.

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