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Original Article

An antiviral drug combinational study against Hepatitis B virus – A computational drug repositioning approach

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ABSTRACT

This study was aimed at identifying two promising generic drugs and conducts an antiviral drug combination studies against Hepatitis B virus. It has two components; computational and *in vitro* studies. The computational study entails sequential screening of all Food and Drug administration-approved drugs (1491) against three protein targets; through structural- and ligand-based pharmacophores screening followed by molecular docking of the selected drugs against the viral targets. Two drugs with the best binding affinities against the viral targets were chosen for an *in vitro* confirmation of activity. The non-toxic concentrations used for the study were established from 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide cytotoxicity study using C_{max} of the drugs as a guide. Iodixanol and sirolimus had the highest binding affinities against the three protein targets. In the antiviral drug combination studies, synergism (Combination index <1) was demonstrated at the three graded concentrations.

Keywords: Antiviral, drug repositioning, ligand-based pharmacophore, molecular docking, pharmacophore modeling, structure-based pharmacophore, viral load

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INTRODUCTION

Infectious diseases are becoming more alarming with high morbidity and mortality in developing countries like Africa.[1] Viral infections like hepatitis B virus (HBV) are often regarded as an incurable and fatal disease.[1] Again, the emergence of drug-resistant strains has compromised the efficacy of most antiviral agents; some have troublesome and unbearable side effects while some are less efficacious.[2,3] Drug repositioning approach might be a better alternative for discovering more effective and less harmful antiviral agents. The high cost of developing drugs has limited the number of antiviral agents into a shortlist.[4]

Viral hepatitis is becoming more alarming with the increasing incidence of chronic viral hepatitis progressing into chronic liver disease and liver cirrhosis. It is estimated that about one-third of the world's population are living with hepatitis B viral infection.[5] Globally, about 400 million people are living with HBV and its the tenth leading cause of death.[5] Twenty-three million Nigerians are currently living with HBV while about 20 million Nigerians are living with both hepatitis C and B viruses.[6] The prevalence of hepatitis B and C in Africa is 10–20% and 6%, respectively.[7] Fewer agents such as interferon, lamivudine, and ribavirin are available for the treatment. These drugs have a spectrum of intolerable side effects and low efficacy.[7]

Several scientists have attempted to reposition approved drugs for the treatment of HBV. For instance, Van de Klundert *et al.*[8] evaluated 640 food and drug administration (FDA)-approved drug for their ability to inhibit HBV transcription in HepG2 transfected cells. Terbinafine, a squalene epoxidase antifungal agent, was found to potently suppress HBx-mediated HBV RNA transcription.

The aim of the study was to identify two promising generic drugs and conduct an antiviral drug combination studies against HBV.

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The objectives of the study include:

- To develop a local database for FDA-approved drugs and three HBV viral protein targets
- To screen commercially approved drugs using structural- and ligand-based pharmacophores as templates
- To run a docking simulation of the selected drugs against the three viral targets and select best two
- To run an *in vitro* antiviral drug combination studies of the two selected drugs against HBV-infected HepG2 cell line.

MATERIALS AND METHODS

Criteria for Viral Targets Selection

The HBV viral targets were selected based on validated selection criteria and scoring.[9] The criteria include: Involvement in a critical pathway necessary for the survival and replication of the viruses, confirmed or putative targets of known antiviral agents, absence of significant cross-talk from the National Center for Biotechnology Information blast search, drugability of the target (easily accessible binding site), and site or location of the protein target within virus; either on the cell surface/cytoplasm or inside the nucleus.[9] Targets with a score of at least 80 out of 115 were selected and considered as critical in the survival and multiplication of the viruses.[10]

Development of Local Database of Viral Protein Targets from Protein Data Bank (PDB)

PDB is an archive of three dimension (3D) structures of about 35,000–50,000 biological molecules. Three HBV viral targets (in PDB text format) necessary for their survival and multiplication were selected and downloaded from the PDB website (www.rcsb.org) and saved in PDB text format. A local database was created for the viral targets in my personal computer.[11]

Development of Local Database for FDA-approved Drug from DrugBank

A DrugBank is a drug database that contains more than 4000 compounds linked to about 14,000 molecular targets. All (1491) FDA-approved drugs were downloaded from DrugBank website (www.drugbank.ca) and saved in structural data format.[12]

Structure-based Pharmacophore Screening

Ligandscout advanced molecular design software was used to generate structure-based pharmacophore for each viral targets using the target cocrystallized ligand complex as a template.[9]

Ligand-based Pharmacophore Screening

Ligand-based pharmacophore was generated using all the cocrystallized ligands for the nine different viral targets as templates. The generated ligand-based pharmacophore can be merged or shared similarity pharmacophore.[9]

Screening of FDA-approved Drugs using the Structural and Ligand-based Pharmacophores as Templates

The structure and ligand-based pharmacophores were copied to the screening perspective using the copy board widget. Approved drugs downloaded from the DrugBank were loaded to the screening database using the "create and load screening database." The generated pharmacophores were screened against the approved drugs and the drugs with similar pharmacophores were displayed in the tabular form compatible with excel.[13]

Docking Simulation of the Selected Drugs from Pharmacophore Screening against Three HBV Viral Targets using PyRx Virtual Screening Tool

Importation of macromolecules from the local database

To import macromolecule from the local database, file > import molecule was selected, this displays "import molecule wizard" carrying different options. Workspace tarball > local file was then selected and "next" button clicked followed by finish button. Shortly an "import completed successfully" dialog appears, then OK button was clicked. The 3D structure of the macromolecule was displayed in the workspace, and the protein ID appears in the "molecule tab" of the navigator panel. Atoms of the macromolecule were viewed in the workspace by deselecting and selecting them in the "molecule tab" of the navigator panel. The macromolecule was inspected in the workspace by right-clicking and holding the mouse. The binding site of the cocrystallized area examined, in shape, size, polarity, and accessibility.[13]

Importation of ligands from the local database

To import ligands from the local database, select open babel button in the control panel of the PyRx tool. Clicking the insert new item tab on the upper left-hand corner of the open babel panel, a "choose open babel supported file" box appears that take you to the ligand database in my personal computer. The ligand of interest was then selected and imported into the PyRx. The selected ligands appear in the open babel results table displaying the drugs ID, formula, weight, and LogP. Minimized atomic coordinates of the ligand were created using "the minimize all" widget. The minimized coordinate of the ligands right-clicked and different options displayed. The option "covert all to AutoDock ligand PDBQT" was selected. The PDPQT format of the ligand appears in the ligand compartment of the AutoDock navigator area.[13]

Running the molecular docking simulation

The ligands of interest were selected from the AutoDock widget and "select ligand" button pressed, followed by the forward button. This automatically inputs the ligands into the ligand list in the control panel of PyRx software. Again, the macromolecules were selected from the AutoDock widget, and "select macromolecule" button pressed followed by the

forward button, and this automatically inputs macromolecules into the macromolecule list in the control panel.[14]

To run Vina, the "run Vina" was clicked, then forward button pressed. Finally, "analyze result" was selected then forward button. This displays the binding affinities of the various poses against the ligands. The lower the binding affinities, the better the protein-ligand interaction since molecules interact to conserve energy.[14]

The analyze results page is where the final docking results were presented. The table was sorted according to the values of the binding energies. The table row was selected one by one to see the corresponding docking pose for each ligand-protein complex in the 3D scene. The numerical results were exported as comma-separated values file compatible with Excel.[14]

Selection of the Best Two (2) Performing Drugs for *In vitro* Antiviral Studies

Two drugs with overall best binding affinities against the three viral targets were selected for confirmation of activity in the wet laboratory using *in vitro* cell line-based assay. The analytical grade of the selected drugs was purchased from Sigma-Aldrich.

In vitro Model for HBV

Ten thousand cells/well were cultured in 24-well plates for 24 h to achieve 80–90% confluency. Media changed and the cells washed with phosphate buffer solution before the addition of viral particles.

- For HepG2 cells, 1 ml of serum infected with HBV (containing 4.7 Log IU/ml of virus) was added to each well.

The infected cells were maintained and propagated in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C in a humidified and 5% CO_2 chamber.[15]

In vitro Antiviral Drug Combination Studies of the Two Selected Drugs

Twenty-four hour post-infection, the HBV infected cell lines were treated with three graded concentrations of the two selected drugs – in combination, dimethyl sulfoxide (DMSO) treated and lamivudine (in duplicates). The viral DNA was released following lyses of the infected cell lines at 48 h post-treatment. The viral loads for each of the treatment groups were quantified using real-time polymerase chain reaction.

Determination of Percentage Viral Inhibition (%VI)

The %VI for each of tested group was calculated using the formula[16]:

$$\%\,VI = \frac{VC - VTG}{VC} \times 100$$

Where VC is the viral load of the DMSO-treated group
VTG is the viral load of the treated group.

Determination of Half-maximal Inhibitory Concentration (IC_{50})

Nonlinear regression analysis curve generated by GraphPad Prism was used to extrapolate the IC_{50} of each drug against each virus. The IC_{50} is the concentration that produces 50% VI.[17]

Determination of Half-maximal Toxic Concentrations (CC_{50})

Nonlinear regression analysis curve generated by GraphPad Prism was used to extrapolate the CC_{50} of each drug against each cell line. The CC_{50} is the concentration that produces 50% cell line viability.[17]

Determination of Selectivity Index (SI)

The SI for each drug against a particular cell line was calculated using the formula.[17]

$$SI = CC_{50}/IC_{50}$$

Determination of the Combination Index (CI) and Dose Reduction Index (DRI)

Compusyn combination software was used to generate the CI and DRI. The (CI) <1 is synergism, CI >1 is antagonism while CI = 1 is additivism. The DRI >1 is favorable, DRI <1 is unfavorable, and DRI = 1 is no dose reduction.[18]

Data Analysis

Data were presented in tables and graphs and were expressed as mean ± SEM. The IC_{50} and CC_{50} were extrapolated from a

Table 1: List of the three selected protein targets and their scores

S/N	Viral protein targets	Critical in the pathway (50)	Putative antiviral drug target (30)	No significant crosstalk (15)	Drug ability of the target (10)	Location of the target in virus (10)	Total Score (115)
1	HBV XIP (3ms6)	50	0	15	10	Cytoplasm (10)	85
2	HBV capsid protein (5d7y)	50	0	15	10	Cytoplasm (10)	85
3	HBV core protein (5t2p)	50	0	15	10	Nucleus (5)	80

XIP: X interacting protein, HBV: Hepatitis B virus

sigmoidal dose-response curve. Statistical differences between the viral loads for the different drug groups and distinct post-treatment time points were analyzed using a two-way ANOVA followed by Dunnett's multiple comparison tests. $P < 0.05$ was considered to be statistically significant. All analyses were performed using GraphPad Prism version 7.

RESULTS AND DISCUSSION

Results

Three selected protein targets and their scores

Nine viral protein targets were identified and validated. The total score for each viral target was >80, hence validated [Table 1].

Selected drugs from structure-and ligand-based pharmacophore

Three hundred and eight (400) FDA drugs and forty-three (40) drugs were selected from the structure- and ligand-based pharmacophore screening.

Best two drugs and there binding affinities

Iodixanol and sirolimus had the best binding affinities against the three HBV targets. The binding affinities for each drug against the viral targets were greater than that for the respective cocrystallized ligands [Table 2].

Table 2: List of best two drugs and their binding affinities

Ligand	HBV XIP	HBV capsid	HBV core
Cocrystallized ligand	−12.0	−10.7	−15.0
Iodixanol	−15.3	−15.4	−19.2
Sirolimus	−16.1	−16.5	−24

Glycoprotein 41, HBV XIP: Hepatitis B virus X interacting protein, HBV capsid: Hepatitis B virus capsid, HBV Core protein: Hepatitis B virus core protein

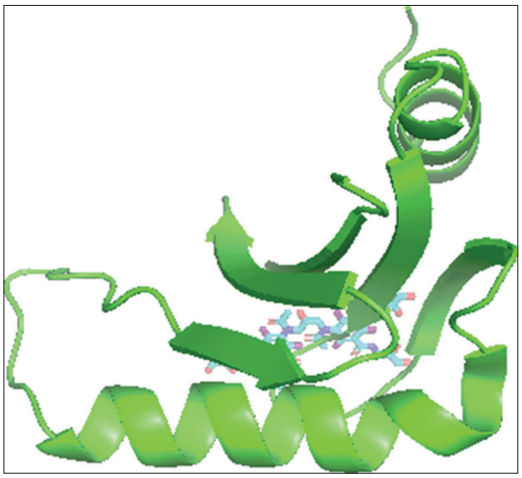

Figure 1: Iodixanol-Hepatitis B virus X interacting protein complex

Iodixanol and sirolimus (ball and stick) superimpose with the cocrystallized ligands (cartoon shape) and fit into the binding poses [Figures 1-6].

Percentage viability of combined iodixanol-sirolimus against HepG2 cell line from 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) cytotoxicity assay

The percentage cell viability was <50% for HepG2 cell lines when treated with iodixanol-sirolimus concentration of >8000/0.8 µg/ml, as shown in Table 3.

Three graded non-toxic concentrations of iodixanol-sirolimus for the in vitro antiviral drug combination studies

The three graded non-toxic concentrations for iodixanol/sirolimus combination selected from MTT cytotoxicity study were used for the antiviral study. The concentrations of the stock solution for each drug and the volume of

Figure 2: Iodixanol-Hepatitis B virus capsid complex

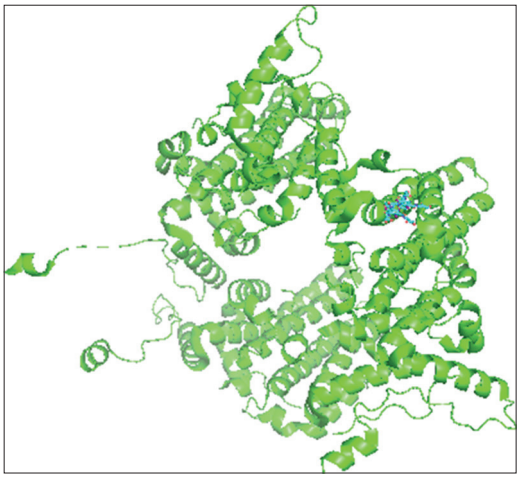

Figure 3: Iodixanol-Hepatitis B virus core protein complex

stock to make the highest concentrations were calculated. This is to allow easy serial dilution during drug treatment [Table 4].

Virucidal effects of iodixanol-sirolimus combination against HBV

At the highest non-toxic concentration, iodixanol-sirolimus produced a statistically significant higher virucidal effect (35%) against HBV compared to lamivudine [Table 5].

SI of the drug combination

Table 6 showed the SI of iodixanol-sirolimus combination against HBV infected cell lines. The higher the SI, the more effective and safe a drug would be during treatment.

Table 3: Percentage viability of combined iodixanol-sirolimus against HepG2 cell lines using MTT cytotoxicity assay

Iodixanol-sirolimus conc.	Percentage viability		
	HBV	HIV	LASSA
1000/0.1 µg/ml	70.08±0.047	70.19±0.01	72.03±0.87
2000/0.2 µg/ml	68.67±0.24	66.25±3.13	67.84±0.47
4000/0.4 µg/ml	64.82±0.32	65.08±0.51	64.28±0.26
8000/0.8 µg/ml	50.12±0.0092	51.09±0.06	51.29±0.26
16,000/1.6 µg/ml	47.34±0.278	48.74±0.05	46.78±0.39

HBV: Hepatitis B Virus, HepG2 cells: Human hepatocellular carcinoma cells, MTT: 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide, Conc.: Concentration, HIV: Human immunodeficiency viruses

Table 4: Three graded non-toxic concentrations of iodixanol-sirolimus for the *in vitro* antiviral drug combination studies

Drugs	Lowest conc. (µg/ml)	Mid conc. (µg/ml)	Highest conc. (µg/ml)
Iodixanol-sirolimus	1000/0.1	2000/0.2	4000/0.4

Conc.: Concentration

CI of iodixanol-sirolimus combination against HBV

As shown in Table 7, the median effect concentration of the combination against HBV was 7370.4 µg/ml which is far less than for iodixanol alone (80678.8 µg/ml). Synergism

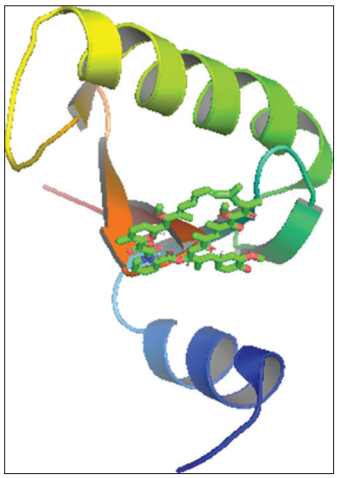

Figure 4: Sirolimus-Hepatitis B virus X interacting protein complex

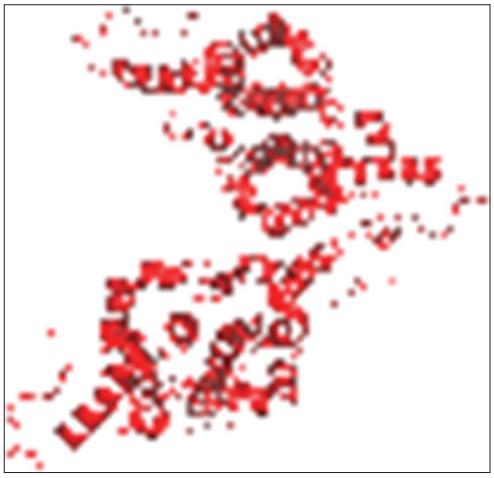

Figure 5: Sirolimus-Hepatitis B virus capsid protein complex

Table 5: Virucidal effects of iodixanol-sirolimus combination against HBV

Drugs	Smallest conc.		Median conc.		Highest conc.	
	Viral load	% VI	Viral load	% VI	Viral load	% VI
HBV						
Iodixanol-sirolimus	3.973±0.014*	13.5	3.391±0.044*	26.2	2.98±0.008***	35.1
Lamivudine	3.944±0.015*	14.2	3.415±0.008*	25.7	3.162±0.000*	31.2
DMSO	4.595±0.009		4.595±0.009		4.595±0.009	

Values are expressed as mean±SEM, n=2, Two-way ANOVA followed by Dunnet's multiple comparison tests was used, Values of the group with superscript *are statistically significant (P<0.05) compared to negative control group, Values of the group with superscript **are statistically significant (P<0.05) compared to positive control group, Values with superscript ***are statistical significant (P<0.05) compared to both negative and positive control groups, %VI: Percentage viral inhibition, HBV: Hepatitis B virus, DMSO: Dimethyl sulfoxide, Conc.: Concentration

was demonstrated at the mid and highest non-toxic concentrations of 2000/0.2 µg/ml and 4000/0.4 µg/ml, respectively.

CI and DRI of iodixanol-sirolimus combination at 50%, 75%, 90%, and 95% VI against HBV

The synergism (CI <1) demonstrated by the drug combination increases with increasing effect levels (percentage effects). The DRI for the drug combination was favorable (DRI >1), and the value increases across the effects levels [Table 8].

Discussion

The study showed that iodixanol-sirolimus combination demonstrated a concentration-dependent HBV killing effect. The virucidal effect (35%) produced against HBV at the highest non-toxic concentration of the drug combination (4000/0.4 µg/ml) was significantly higher than the effect (31.2%) produced by lamivudine alone. Remarkably, synergism (CI <1) was demonstrated at mid and highest non-toxic concentrations of 2000/0.2 µg/ml and 4000/0.4 µg/ml. Again, the median effective concentration of the drug combination was 7370.4 µg/ml, by far less than for iodixanol alone (80,678.8 µg/ml), indicating the high potency of the drug combination. In addition, the DRI for the drug combination was favorable (i.e., DRI >1) in the treatment for HBV further buttressing the established synergistic effect

Table 6: SI for iodixanol and sirolimus combination in cell-line infected viruses

Iodixanol-sirolimus	HBV
CC_{50}	11020
IC_{50}	7731
SI	1.40

HBV: Hepatitis B virus, CC_{50} is the concentration that produces 50% cell line viability, IC_{50} is the concentration that produces 50% viral inhibition, SI = CC_{50}/IC_{50} SI: Selectivity index, Both IC_{50} and CC_{50} were extrapolated using nonlinear regression analysis generated by GraphPad Prism

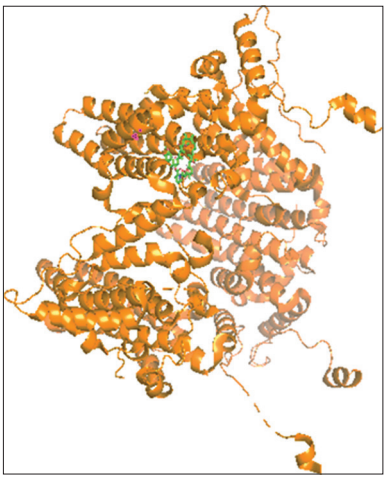

Figure 6: Sirolimus-Hepatitis B virus core protein complex

Table 7: Concentration effect (%VI) relationship of iodixanol, sirolimus, and the drug combination against hepatitis B virus infected HepG2 cell line

Drug (µg/ml)		Parameter			R	CI
Iodixanol	Sirolimus	Fractional inhibition (fa)	M	Dm (µg/ml)		
D1						
1000		0.125				
2000		0.162				
4000		0.209	0.44359	80678.8	0.99996	
	D2					
	0.1	0.121				
	0.2	0.193				
	0.4	0.229	0.55474	3.2259	0.96968	
D1+D2 (10000:1)						
1000+0.1		0.135				1.69825
2000+0.2		0.262				0.65695
4000+0.4		0.351	0.8965	7370.4	0.9831	0.57368

CI values of <1, =1, and >1 indicate synergism, additive, and antagonism, respectively. CI: Combination index, M: The slope of the median effect dose; M=1, >1, and <1 indicates hyperbolic, sigmoidal, and flat sigmoidal, respectively, D1: Doses of Iodixanol, D2: Doses of Sirolimus, Fa: Fractional inhibition is the virucidal effect in fraction of ≤1, Dm: The median effect dose or the IC50. It signifies the potency of the drug, R: The linear correlation coefficient of the mean effect plot. It signifies the conformity of the data with the mass action law. Usually, *r*>0.9 are considered good. %VI: Percentage viral inhibition

Table 8: CI and DRI of iodixanol-sirolimus combination at 50%, 75%, 90%, and 95% viral inhibition against HBV

Virus	Drug combination	CR	CI values at inhibition of				DRI values at inhibition of			
			50%	75%	90%	95%	50%	75%	90%	95%
HBV	Iodixanol+sirolimus	10000:1	0.3198	0.13352	0.05795	0.0334	10.9474	38.2557	133.684	313.083
							4.3773	9.31292	19.8137	33.1109

HBV: Hepatitis B virus. CI values of <1, =1, and >1 indicate synergism, additive, and antagonism, respectively. DRI values of <1, =1, and >1 Not favorable DR, No DR, and Favorable DR, respectively, CI: Combination index. DRI: Dose reduction index

of the drug combination. Interestingly, the SI for the drug combination against HBV infected cell line was 1.4, slightly >1.3, 0.9, and 1.0 for lamivudine, iodixanol, and sirolimus, respectively.

From the literature search using different search parameters, this study was the first to identify the anti-HBV effect of sirolimus. However, sirolimus an inhibitor of mammalian target of rapamycin (mTOR) was found to be a potent inhibitor of Hepatitis C virus (HCV) RNA replication using Huh-7.5 cells and primary human hepatocytes.[19] Again, in 42 HCV-infected liver-transplanted and kidney-transplanted patients who were switched to an mTOR inhibitor, decrease HCV RNA loads were reported.[20] It is also an established fact that efficient HCV RNA replication is dependent on the presence of the mTORC1 signaling pathway.[21] Sirolimus is also a central regulator of gene expression, translation, and various metabolic processes.[22]

From the literature search using different search parameters, no known study has reported the anti-HBV effect of iodixanol. However, copper iodide, an iodine-containing compound exert antiviral activity against H1N1 influenza by generating hydroxyl radicals.[23] Similarly, Povidone-iodine solution showed good efficacy against both enveloped and non-enveloped viruses including adenovirus and polyomaviruses.[24] Conflictingly, an antimicrobial study revealed iodixanol not to impede bacteria growth in a culture media.[25]

CONCLUSION

The result of the study showed that iodixanol-sirolimus combination produced a concentration-dependent viral killing against HBV. The low potency and SI of the two drugs against the infected cell line are the issue of concern. Synergism was demonstrated against HBV.

It can be concluded that there exist generic drugs with activity against HBV.

Further evaluation of the iodixanol and sirolimus against the three viruses using different cell lines followed by *in vivo* studies at biosafety level IV is recommended.

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