IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 6 Issue 8, August 2019
ISSN (Online) 2348 – 7968
www.ijiset.com

An antiviral drug Combinational Studies Against Lassa Virus- A Computational Drug Repositioning Approach.

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Abstract

This study was aimed at identifying two promising generic drugs and conduct an antiviral drug combination studies against Lassa virus. It has two components; computational and *in-vitro* studies. The computational study entails sequential screening of all FDA approved drugs (1491) against three protein targets; through structural- and ligand- based pharmacophore screening followed by molecular docking of the selected drugs against the viral targets. Two (2) 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 MTT cytotoxicity study using C_{max} of the drugs as a guide. Iodixanol and sirolismus had the highest binding affinities against the three protein targets. In the antiviral drug combination studies, antagonism (CI>1) were demonstrated at the three graded concentrations.

Key words: Molecular docking, Pharmacophore-modelling, structure based pharmacophore, ligand based pharmacophore, drug-repositioning, antiviral, viral load.

1.0 Introduction

Infectious diseases are becoming more alarming with high morbidity and mortality in developing countries like Africa (Yamani et al., 2017). Viral infections like Lassa virus are often regarded as incurable and fatal diseases (Yamani et al., 2017). Again, the emergence of drug resistant strains have compromised the efficacy of most antiviral agents; some have troublesome and unbearable side effects while some are less efficacious (Kühnert et al., 2018; Wang et al., 2018). 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 short list (Bhandari, 2017). Lassa fever is a deadly hemorrhagic disease of great public health importance (Akpede et al., 2018). The Lassa fever outbreak in Nigeria in 2018 was alarming, as 428 cases with 107 mortalities were recorded within the period of 5 months from January to May, 2018 (Tambo et al., 2018). This was the largest outbreak of Lassa fever (case fatality of 25%) ever reported in Nigeria. Ribavirin, a must win, is the only available drug for Lassa fever.

1.1 Aim of the Study

To identify two promising generic drugs and conduct an antiviral drug combination studies against Lassa virus.

1.2 Objectives of the Study

- To develop a local database for FDA-Approved drugs and three (3) Lassa 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 (2) selected drugs against Lassa infected Caco-2 cell line.

2.0 Materials and Methods

2.1 Criteria for Viral Targets Selection

The Lassa viral targets were selected based on validated selection criteria and scoring (Ansari et al., 2018). 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 NCBI 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 (Sliwoski et al., 2014; Ansari et al., 2018). Targets with score of at least 80 out of 115 were selected and considered as critical in the survival and multiplication of the viruses (Sliwoski et al., 2014).

2.2 Development of Local Database of Viral Protein Targets from Protein DataBank

Protein Data Bank (PDB) is an archive of 3D structures of about 35,000-50,000 biological molecules. Three (3) Lassa 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 (Smart et al., 2018).

2.3 Development of Local Database for FDA-Approved Drug from DrugBank

A DrugBank is a drug Database that contains more than 4,000 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 (SDF) (Wishart et al., 2017).

2.4 Structure-based Pharmacophore Screening

Ligand Scout advanced molecular design software was used to generate structure-based pharmacophore for each viral targets using the target- co-crystalized ligand complex as a template (Ansari et al., 2018).

2.5 Ligand-Based Pharmacophore Screening

Ligand-based pharmacophore was generated using all the co-crystalized ligands for the three different viral targets as templates. The generated ligand-based pharmacophore can be merged or shared similarity pharmacophore (Ansari et al., 2018).

2.6 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 (Ansari et al., 2018).

2.7 Docking Simulation of the Selected Drugs from Pharmacophore Screening against three (3) Lassa Viral Targets using PyRx Virtual Screening Tool

2.7.1 Importation of macromolecules from the local Database

To import macromolecule from 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 co-crystallized area examined, in shape, size, polarity and accessibility (Ellingson and Baudry, 2014).

2.7.2 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 takes 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 was 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 (Ellingson and Baudry, 2014).

2.7.3 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 input 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 input macromolecules into the macromolecule list in the control panel (Ellingson and Baudry, 2014).

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 (Dallakyan and Olson, 2015).

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 a Comma-Separated Values (CSV) file compatible with excel (Dallakyan and Olson, 2015).

2.7.4 Selection of the Best two (2) Performing Drugs for In-vitro Antiviral Studies

Two (2) 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 were purchased from sigma Aldrich.

2.7.5 In-vitro Model for Lassa Virus

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

- For Caco-2 cells, 1ml of infected supernatant containing 0.092 LogIU/ml of Lassa virus was added to each well.

The infected cells were maintained and propagated in DMEM (Dulbecco's Modified Eagle Medium) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin and incubated at 37°C in a humidified and 5% CO_2 chamber (Farag and Mansour, 2016).

2.7.6 In-vitro Antiviral drug combination studies of the Two Selected Drugs

Twenty four hour post infection, the Lassa infected cell lines were treated with three graded concentrations of the two selected drugs-in combination, DMSO treated and ribavirin (in duplicates). The viral RNA were released following lyses of the infected cell lines at 48 hours post treatment.

2.8 Determination of percentage viral inhibition (%VI)

The percentage viral inhibition for each of tested group was calculated using the formula (Kati et al., 2015):

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

Where VC is the viral load of the DMSO treated group

VTG is the viral load of the treated group.

2.9 Determination of IC_{50}

Nonlinear regression analysis curve generated by graph pad prism was used to extrapolate the IC_{50} of each drug against each virus. The IC_{50} is the concentration that produces 50% viral inhibition (Lo et al., 2016).

2.10 Determination of CC_{50}

Nonlinear regression analysis curve generated by graph pad 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 (Lo et al., 2016).

2.11 Determination of Selectivity Index (SI)

The selectivity index for the combined drugs against Caco-2 cell line was calculated using the formular (Lo et al., 2016).

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

2.12 Determination of the Combination Index (Cl) and Dose Reduction Index (DRI)

Compusyn combination software was used to generate the combination index and Dose reduction index. 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 (Ashton, 2015).

2.13 Data Analysis

Data were presented in tables and graphs and were expressed as mean ±SEM. The half- maximal inhibitory concentration (IC_{50}) and half-maximal toxic concentrations (CC50) were extrapolated from a sigmoidal dose-response curve. Statistical differences between the viral loads for the different drug groups and distinct post treatment time points were analyze using a two-way ANOVA followed by Dunnet's multiple comparison tests. The *p* values of < 0.05 were considered to be statistically significant. All analyses were performed using Graph Pad Prism version 7. Compusyn software was used to calculate the combination index (Cl) and dose reduction index (DRI) for each of the drug combination.

3.0 Results

3.1 Three (3) Selected Protein Targets and their Scores

Three viral protein targets were identified and validated. The total score for each viral target was greater than 80, hence validated (Table 1).

Table 1: Three (3) Selected Protein Targets and their Scores

S/N	Viral protein targets	Critical in the pathway (50)	Putative Antiviral drug target (30)	No signi-ficant crosstalk (15)	Drug ability of the target (10)	Location of target in virus (10)	Total Score (115)
7	Lassa nucleo-protein (3mx5)	50	0	15	10	Surface(10)	85
8	Lassa glycol-protein (4zjf)	50	0	15	10	Surface(10)	85
9	Lassa polymerase (5j1n)	50	30	15	10	Nucleus (5)	110

3.2 Selected Drugs from Structure-based Pharmacophore

Three hundred and eight (209) FDA drugs and fourty three (20) drugs were selected from the structure- and ligand-based pharmacophore screening.

3.3 Best Two (2) Drugs and there Binding Affinities

Iodixanol and sirolismus had the best binding affinities against the three Lassa targets. The binding affinities for each drug against the viral targets were greater than that for the respective co-crystalized ligands (Table 2).

Table 2: List of Best Two (2) Drugs and there Binding Affinities

Ligand	Lassa nucleo protein	Lassa glycol protein	Lassa polymerase
Co-crystalized ligand	-14.5	-10.5	-9.9
Iodixanol	-21.9	-15.2	-16.5
Sirolismus	-20.4	-18.3	-18.6

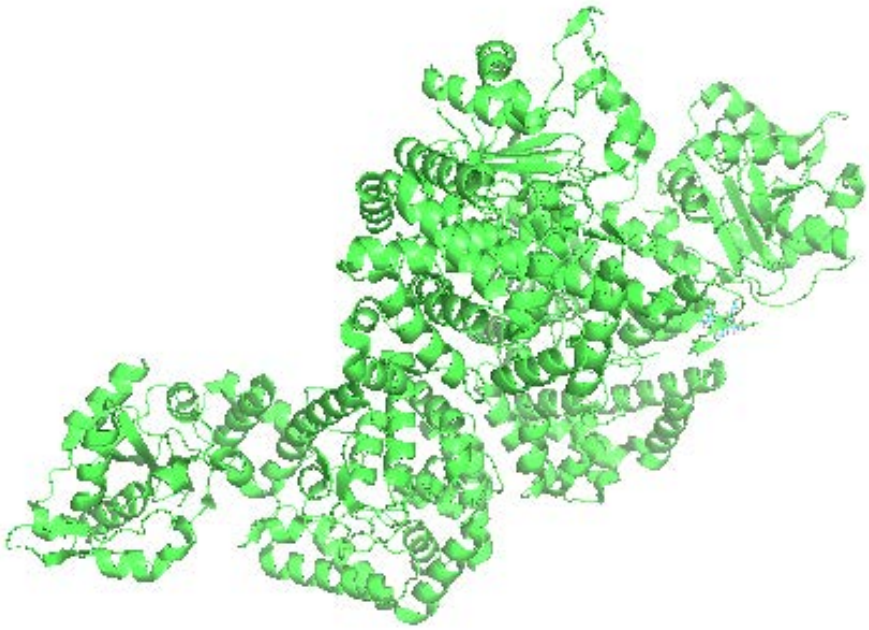

Figure 1: Iodixanol- Lassa Nucleoprotein Complex

Figure 2: Iodixanol- Lassa Glycoprotein Complex

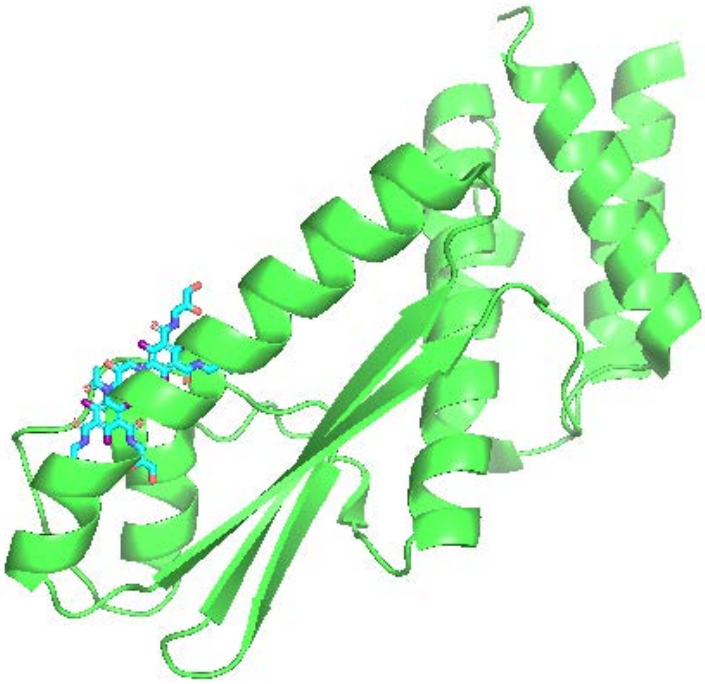

Figure 3: Iodixanol- Lassa Polymerase Complex

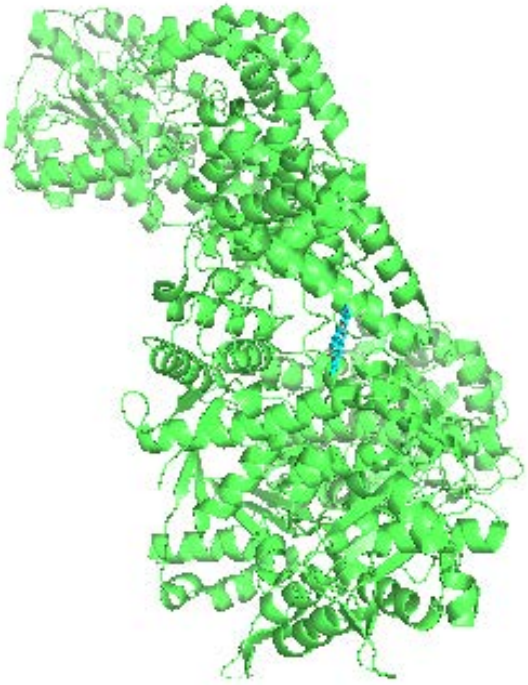

Figure 4: Sirolismus-Lassa Nucleoprotein Complex

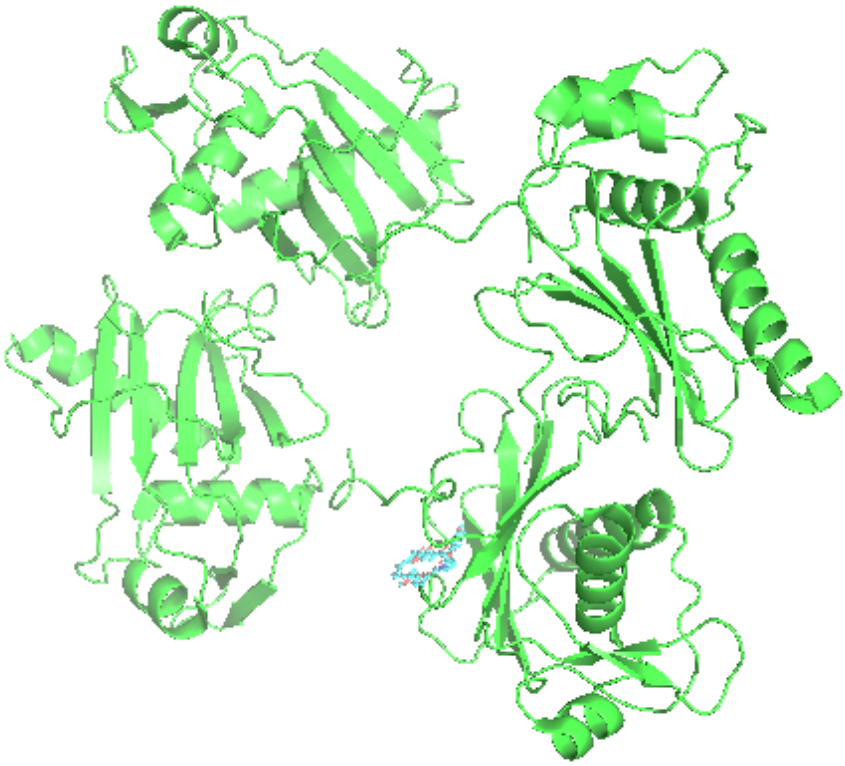

Figure 5: Sirolismus-Lassa Glycoprotein Complex

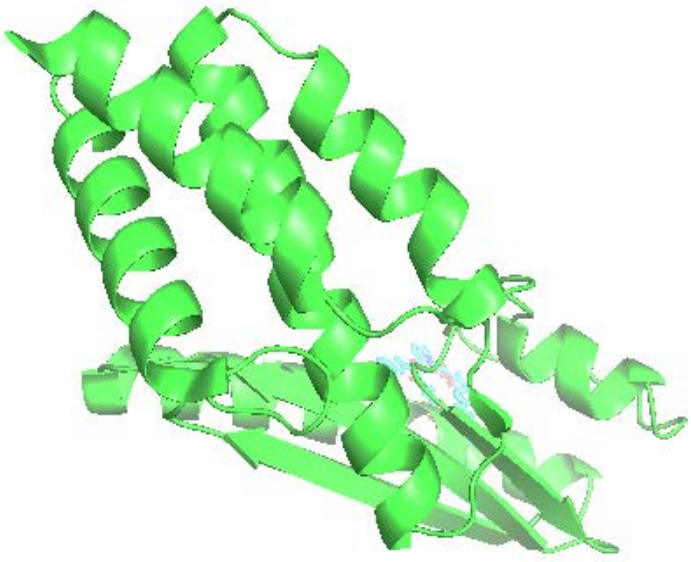

Figure 6: Sirolismus-Lassa Polymerase Complex

Figure 1-6: Sirolismus and iodixanol (ball and stick) superimposes with the co-crystalized ligands (cartoon shape) and fit into the binding poses.

3.4 Percentage Viability of combined Iodixanol-Sirolimus against Caco-2 Cell Lines Using MTT Cytotoxicity Assay

The percentage cell viability were less than 50% when treated with iodixanol/sirolismus concentration of greater than 8000/0.8µg/ml as shown in Table 3.

Table 3: Percentage Viability of combined Iodixanol-Sirolimus against Caco-2 Cell Lines Using MTT Cytotoxicity Assay

IODIXANOL- SIROLISMUS CONC.	LASSA
1000/0.1µg/ml	72.03±0.87
2000/0.2µg/ml	67.84±0.47
4000/0.4µg/ml	64.28±0.26
8000/0.8µg/ml	51.29±0.26
16000/1.6µg/ml	46.78±0.39

Keys:
Caco-2 cells- Human epithelia colorectal adenocarcinoma cells
MTT- 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide
CONC. Concentration

3.5 Three Graded nontoxic concentrations of Iodixanol-Sirolismus for the In vitro Antiviral Drug Combination Studies

The three graded non- toxic concentrations for iodixanol/sirolismus 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 stock to make the highest concentrations were calculated. This is to allow easy serial dilution during drug treatment (Table 4).

Table 4: Three Graded nontoxic concentrations of Iodixanol-Sirolismus for the In vitro Antiviral Drug Combination Studies

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

3.6 Virucidal Effects of Iodixanol-Sirolimus combination against Lassa-virus

At highest non-toxic concentration, ribavirin produced a statistically significant higher virucidal effect (54.9%) against Lassa virus compared to the drug combination (31.9%), See Table 5.

Table 5: Virucidal Effects of Iodixanol-sirolismus combination against Lassa-virus at Three Graded Concentrations

		Smallest Conc.		Median Conc.		Highest Conc.	
	Drugs	Viral load	%VI	Viral load	%VI	Viral load	%VI
Lassa	Iodixanol-sirolismus	0.079 ±0.002***	13.2	0.074 ± 0.003***	18.7	0.062± 0.003***	31.9
	Ribavirin	0.062 ± 0.001*	31.9	0.049 ±0.002*	46.2	0.041±0.002*	54.9
	DMSO	0.091 ± 0.000		0.091±0.000		0.091±0.000	

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.
The higher %VI the more the antiviral activity of the drug.
Keys: HBV- Hepatitis B Virus, HIV- Human Immunodeficiency Virus, DMSO = Dimethyl sulfoxide (Negative control), %VI = VC-VTG/VC X 100
%VI = Percentage viral inhibition, VC = viral load of the DMSO treated group
VTG = Viral load of the drug treated group, Conc. Concentration

4.27 Selectivity Index (SI) of the Drug Combination

Table 21 showed the selectivity index (SI) of iodixanol-sirolismus combination against Lassa-virus infected cell lines. The higher the SI the more effective and safe a drug would be during treatment.

Table 6: Selectivity index (SI) for Iodixanol and Sirolismus Combination in Cell-Line Infected Viruses

IODIXANOL-SIROLISMUS	LASSA
CC_{50}	10904
IC_{50}	10483
SI	1.00

Key:
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 graph-pad prism.

3.7 Combination Index (CI) of Iodixanol-Sirolismus combination against Lassa-virus

As shown in Table 24, the median effect concentration of the combination against Lassa was 10826.4 µg/ml which is far greater than 3802.18 of iodixanol alone. Antagonism were demonstrated at the three graded concentrations used for the study.

Table 7: Concentration-Effect (%VI) Relationship of Iodixanol, Sirolismus and drug Combination against Lassa infected Caco-2 Cell Line

Drug(µg/ml)		Parameter				
Iodixanol	Sirolismus	Fractional inhibition (fa)	m	Dm(µg/ml)	R	CI
D1						
1000		0.055				
2000		0.395				
4000		0.429	1.84515	3802.18	0.8894	
	D2					
	0.1	0.11				
	0.2	0.407				
	0.4	0.505	1.52258	0.34174	0.94074	
D1+D2 (10000:1)						
1000+0.1		0.132				1.73802
2000+0.2		0.187				2.70301
4000+0.4		0.319	0.81153	10826.4	0.98855	3.5129

CI values of <1, =1and >1 indicate synergism, additive and antagonism respectively.
Keys:
Caco-2 cells – Human epithelia colorectal adenocarcinoma cells
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 Sirolismus
Fa- Fractional inhibition is the virucidal effect in fraction of less than or equal to 1
Dm- The median effect dose or the IC_{50}. 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.

3.8 Combination index (CI) and Dose reduction index (DRI) of Iodixanol -Sirolismus combination at 50%, 75%, 90% and 95% Viral Inhibition against Lassa-virus

The antagonism (CI>1) demonstrated by the drug combination increases with increasing effect levels (percentage effects). The dose reduction index (DRI) for both iodixanol and sirolismus were Non-favourable (DRI<1) and the value decreases across the effects levels (Table 8).

Table 8: Combination index (CI) and Dose reduction index (DRI) of Iodixanol-Sirolismus Combination at 50%, 75%, 90% and 95% Viral Inhibition against Lassa-virus

			CI values at inhibition of				DRI values at inhibition of			
Virus	Drug combination	CR	50%	75%	90%	95%	50%	75%	90%	95%
LASSA	Iodixanol + Sirolismus	10000:1	6.0148	12.0387	24.1916	38.9752	0.35123	0.16453	0.07707	0.04601
							0.31569	0.16776	0.08915	0.05799

CI values of <1, =1and >1 indicate synergism, additive and antagonism respectively.
. DRI values of <1, =1 and >1 Not favourable DR, No DR and Favourable DR respectively
Keys:
CI- Combination Index
DRI- Dose reduction index
CR- Combination ratio

4.0 Discussion

The study showed that iodixanol-sirolismus combination demonstrated a concentration dependent Lassa viral killing effects. The highest virucidal effect (31.9%) produced by the drug combination against Lassa was significantly less than that produced by ribavirin (54.9%). Contrarily, antagonism (CI>1) were demonstrated at the three graded concentrations used for the study against Lassa and the antagonism increases with increasing effect levels. Again, the median effective concentration of the combination against Lassa was 10,826.4 µg/ml, three times that for iodixanol (3,802.18) alone, indicating the low potency for the drug combination.

Additionally, the dose reduction index (DRI) for the drug combination was non- favourable (i.e DRI less than 1) in the treatment for Lassa, again buttressing the established antagonistic effect of the drug combination. Again, the selectivity index for the drug combination against Lassa infected cell line was 1, less than 6, 3.2 and 2.5 for ribavirin, iodixanol and sirolismus respectively. The findings that iodixanol and sirolismus produce statistically significant virucidal effects (49.6% and 49.7% respectively) against Lassa virus is interesting, but that this did not translate to synergistic interaction is worrisome. A possible explanation might be that, the drugs share the same binding site on the Lassa virus nucleoprotein, hence compete for attachment site.

From the literature search using different search parameters, this study was the first to identify the anti-Lassa effect of sirolismus and iodixanol. However, previous study revealed that solid-organ transplant recipients treated with mTOR inhibitors; sirolimus or everolimus, have fewer clinical Cytomegalovirus (an envelope virus like Lassa) infections than those on other forms of immunosuppression (Nashan, 2018).

From the literature search using different search parameters, no known study have reported anti-Lassa effect of iodixanol. However, copper iodide, an iodine-containing compound exert Antiviral activity

against H1NI influenza by generating hydroxyl radicals (Vincent et al., 2016). Similarly, Povidone iodine solution showed good efficacy against both enveloped and non- enveloped viruses including adenovirus and polyomaviruses (Eggers et al., 2015). Conflictingly, an antimicrobial study revealed iodixanol not to impede bacteria growth in a culture media (Klimentová and Stulík, 2015).

5.0 Conclusion

The result of the study showed that iodixanol-sirolismus combination produced a concentration dependent viral killing with demonstrated antagonism.

5.1 Recommendations

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

5.2 Limitations of the study

Because of time limit and financial constraints, this study focus mainly on the measurement of virucidal effect using real time PCR. Combining different methods gives a more robust and validated findings. Other methods include;

(1) Flow cytometry measurement of specific viral proteins/antigens in the infected supernatant (like Lassa polymerase) by flow cytometry.
(2) Measurement of viral induced cytopathic effects via cell counting and microscopy.

Acknowledgement

My gratitude goes to Dr. Mustapha Imam and the technical staff of Dna lab, Kaduna, Nigeria for their immense efforts in fashioning and standardizing this work.

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