MethodsX 8 (2021) 101381

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Method Article

The profiling, identification, quantification and analysis of differentially expressed genes (DEGs) in response to drug treatment in lung cancer

Rahaba Marima[a,b,*], Rodney Hull[a], Zodwa Dlamini[a], Clement Penny[b]

[a] SAMRC/UP Precision Prevention and Novel Drug Targets for HIV-Associated Cancers Extramural Unit, Pan African Cancer Research Institute, Faculty of Health Sciences, University of Pretoria, Hatfield 0028, South Africa
[b] Department of Internal Medicine, School of Medicine, Faculty of Health Sciences, University of the Witwatersrand, Parktown, 2193, South Africa

A B S T R A C T

The profiling and identification of genes that are differentially expressed is frequently used to underpin the underlying molecular mechanisms of biological conditions and provides a molecular foothold on biological questions of interest. However, this can be a daunting task since there is a cross talk and overlap of some of the components of the signalling pathways. The deregulation of the cell cycle signalling pathway is a hallmark of cancer, including lung cancer. Proper regulation of the cell cycle results in cellular homeostasis between cell proliferation and cell death. The comprehension of the cell cycle regulation in drug metabolism studies is of significance. This study aimed at elucidating the regulation of cell cycle genes' in response to LPV/r in lung cells. Thus, this study describes methodology for revealing molecular mechanisms employed by LPV/r to induce stress on genomic DNA. This approach is based on the interrogation of a panel of 84 genes related to the cell cycle pathway, and how the differentially expressed genes' expression pattern corroborates loss in nuclear integrity (phenotypic observation). MAD2L2, AURKB and CASP3 gene expressions were further confirmed by RT-qPCR. Furthermore, the use of *in-silico* bioinformatics tools integrates the molecular profiles and phenotypic changes. This approach revealed the activation of the DNA damage response (DDR) pathway in response to LPV/r treatment. The proposed methodology will aid in the comprehension of drug metabolism at genotypic and phenotypic levels.

- Gene profiling often reveals the underlying molecular mechanisms.
- RT^2 PCR gene arrays have integrated patented quality controls and allow reliable gene expression analysis.
- *In-silico* bioinformatics analysis help reveal pathways affected, that often correspond to phenotypic changes/features.

© 2021 The Author(s). Published by Elsevier B.V.
This is an open access article under the CC BY-NC-ND license
(http://creativecommons.org/licenses/by-nc-nd/4.0/)

DOI of original article: 10.1016/j.biopha.2020.110829
* Corresponding author.
E-mail address: rahaba.marima@up.ac.za (R. Marima).

https://doi.org/10.1016/j.mex.2021.101381
2215-0161/© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

ARTICLE INFO
Method name: PCR gene arrays
Keywords: Lopinavir/ritonavir (LPV/r), MRC-5 cells, A549 cells, Drug treatment, Differential gene expression (DEG), Ingenuity Pathway Analysis (IPA)
Article history: Received 15 October 2020; Accepted 2 May 2021; Available online 11 May 2021

Specifications table

Subject Area	*Biochemistry, Genetics and Molecular Biology*
More specific subject area	*Molecular Oncology*
Protocol name	*PCR gene arrays*
Reagents/tools	*RT^2 Profiler Human PCR Arrays (Qiagen PAHS-020Z)-Cell Cycle*
Experimental design	*Human lung cells were cultured, split and synchrosined prior to LPV/r drug treatment for nuclear integrity interrogation and gene expression profiling and analysis.*
Trial registration	*N/A*
Ethics	*N/A.*
Value of the Protocol	• Gene profiling often reveals the underlying molecular mechanisms • RT^2 PCR gene arrays have integrated patented quality controls and allow reliable gene expression analysis • *In-silico* bioinformatics analysis help reveal pathways affected, that often corresponds to phenotypic changes/features
Biochemistry, Genetics and Molecular Biology	

Introduction

Lung cancer is one of the leading cause of deaths in both men and women, globally and in South Africa [1,2]. The increase in lung cancer incidence rates is particularly observed in the HIV population. As a non-AIDS defining cancer (NADC), lung cancer is rising as a colliding epidemic with HIV. While antiretroviral (ARV) treatment improves the health related quality of life (HRQoL) for HIV positive patients on ARV treatment, the rise in lung cancer cases in the ARV treatment (ART) era remains to be fully elucidated [3]. While the cell cycle is a tightly regulated process, anti-cancer drugs targeting key role players in cell cycle regulation, cell cycle checkpoints and DNA damage response continue to emerge [4–6]. Lopinavir/ritonavir (LPV/r) is a WHO approved dual protease inhibitor used against HIV infection [7]. Previous reports revealed the cytotoxic properties of this protease inhibitor against cancer cells. However, the effects of LPV/r treatment in non-cancerous healthy cells remains elusive . Nelfinavir is also one of the PIs showing cytotoxic effects [8,9]. Although LPV/r is a well established PI has been approved for HIV/AIDS treatment as per [7] guidelines, this study reveals how novel anti-cancer drugs could be mechanistically evaluated in vitro for cytotoxicity. Such novel work can include the eco-friendly preparation of nanomaterials and investigation of their properties in pharmaceutical and medical applications or cancer therapeutics as well as energy and environmental remediation [10–17]. This study aimed at determining the changes in the expression of cell cycle related genes' in response to LPV/r in normal healthy and adenocarcinoma lung cells. This study employed a human cell cycle 84-gene array panel, accompanied by pathway analysis and nuclear interrogation approaches.

Method details

Cell culture

The lung cell lines MRC-5 (normal lung fibroblast (ATCC CCL171) and A549 (lung adenocarcinoma (ATCC CCL185) were purchased from the American Type Culture Collection (ATCC). These cell lines are adaptable to a single growth medium and they have been previously demonstrated to show reproducible profiles for growth and drug sensitivity. MRC-5 and A549 cell lines were treated

with a range of concentrations, including the plasma concentration level, of LPV/r at 24h and 48h time intervals, for screening purposes. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM):F12, 10% serum and 1% pen/strep and left to grow to ~70% confluency in a humid chamber at 37°C and a 5% CO_2 level for 48h to 72h. This was followed by trypsinization, to detach cells from the flask, centrifuging to pellet the cells and remove the supernatant, washing with PBS and re-suspending the cells in 5ml of fresh DMEM media. (For gene expression studies, cells' scraping can sometimes yield improved nucleic acid amounts, as indicated under RNA extraction). The cells were split in a 1:2 ratio and sub-cultured. Cell culture medium containing 10% FBS and 1% pen/strep was replaced every 2-3 days until cells reached 70-80% confluency. Cells were then serum-starved for 24h to synchronise the cell cycle. The following day, the cells were pharmacologically treated with LPV/r at 32 and 80 µM, for 24h to 48h. Vehicle control cells were exposed to growth medium and vehicle only (methanol 0.1% v/v).

DNA staining using 4, 6-diamidino-2-phenylindole (DAPI)

A variety of fluorescent dyes are available to label DNA and allow for easy visualization of the nucleus in interphase cells and chromosomes in mitotic cells. These stains include Hoechst, 4,6-diamidino- 2-phenylindole (DAPI), ethidium bromide, propidium iodide, and acridine orange. Compared to the Hoechst DNA staining, DAPI has greater photostability [18]. It is assumed that DAPI associates with the minor groove of double-stranded DNA, with a preference for the adenine-thymine clusters. For DAPI to enter the cell and intercalate into DNA, cells must be first permeabilized with a detergent before or after fixation. Fluorescence increases approximately 20-fold when DAPI is bound to double-stranded DNA. Here, cells (MRC-5 and A549) were treated for 48h with LPV/r at 32 and 80 µM. The two concentrations were selected as a plasma relevant and an experimental dose. DAPI was used to assess the effects of the ARVs on the cellular DNA using the following protocol:

One (1) $\times$ 10^4 cells were seeded on the coverslips and allowed to grow overnight in 6-well plates. Cells were then rinsed with PBS and serum-starved for 24h. This was then followed by the treatment of cells with 32 µM and 80 µM of LPV/r. A 1 $\times$ PBS would be required for the preparation of DAPI solution. The DAPI (Millipore-Sigma) solution was then diluted 1:5000 in PBS for staining in the later steps. Next, the cell culture medium was removed from the cells grown on the coverslips, the cells were than washed three time using PBS. The cells were fixed for 10min in 3.7% formaldehyde in PBS. The fixative was then removed and the cells were rinsed three times, 5min each, in PBS. The cells were then permeabilized in 0.2% Triton X-100 in PBS for 5min. The triton-X was then aspirated, and the cells were rinsed three times, 5min each, in PBS. Cells were then incubated for 10min at room temperature with 100 µl of the diluted DAPI labelling solution The. DAPI was then removed and the cells were rinsed three times in PBS. The coverslips were then mounted with aqueous FluoroMount mounting medium (Sigma) and allowed to dry. The cells were then viewed on a Zeiss LSM 780 confocal microscope.

Human Cell-Cycle Gene (PCR) Arrays

A gene array panel was used to analyze the cell-cycle response pre- and post- LPV/r treatments of each cell-line. This method provides for a highly reliable and sensitive gene expression profiling of focused panels of genes in signal transduction, biological processes, or disease research pathways using real-time PCR methodology. In this study, RT^2 Profiler Human Cell-cycle PCR Arrays (PAHS-020Z) were used (Qiagen). This pathway-focused array consists of 84 genes related to the cell-cycle, in a 96-well plate format as illustrated in Table 1. Aside from the pathway-focused genes, the panel contains 5 housekeeping (reference) genes and additionally a panel of proprietary controls that monitor genomic DNA contamination (GDC), first strand synthesis (RTC) and real-time PCR efficiency (PPC). Use of such a panel requires the isolation of high quality cellular RNA and sufficient cDNA synthesis to provide for the PCR amplification and analysis of gene expression. These steps are described below.

Table 1
The Human cell cycle PCR gene array layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABL1	ANAPC2	ATM	ATR	AURKA	AURKB	BCCIP	BCL2	BIRC5	BRCA1	BRCA2	CASP3
	NM_005157	NM_013366	NM_000051	NM_001184	NM_003600	NM_004217	NM_016567	NM_000633	NM_001168	NM_007294	NM_000059	NM_004346
B	CCNA2	CCNB1	CCNB2	CCNC	CCND1	CCND2	CCND3	CCNE1	CCNF	CCNG1	CCNG2	CCNH
	NM_001237	NM_031966	NM_004701	NM_005190	NM_053056	NM_001759	NM_001760	NM_001238	NM_001761	NM_004060	NM_004354	NM_001239
C	CCNT1	CDC16	CDC20	CDC25A	CDC25C	CDC34	CDC6	CDK1	CDK2	CDK4	CDK5R1	CDK5RAP1
	NM_001240	NM_003903	NM_001255	NM_001789	NM_001790	NM_004359	NM_001254	NM_001786	NM_001798	NM_000075	NM_003885	NM_016408
D	CDK6	CDK7	CDK8	CDKN1A	CDKN1B	CDKN2A	CDKN2B	CDKN3	CHEK1	CHEK2	CKS1B	CKS2
	NM_001259	NM_001799	NM_001260	NM_000389	NM_004064	NM_000077	NM_004936	NM_005192	NM_001274	NM_007194	NM_001826	NM_001827
E	CUL1	CUL2	CUL3	E2F1	E2F4	GADD45A	GTSE1	HUS1	KNTC1	KPNA2	MAD2L1	MAD2L2
	NM_003592	NM_003591	NM_003590	NM_005225	NM_001950	NM_001924	NM_016426	NM_004507	NM_014708	NM_002266	NM_002358	NM_006341
F	MCM2	MCM3	MCM4	MCM5	MDM2	MKI67	MNAT1	MRE11A	NBN	RAD1	RAD17	RAD51
	NM_004526	NM_002388	NM_005914	NM_006739	NM_002392	NM_002417	NM_002431	NM_005590	NM_002485	NM_002853	NM_002873	NM_002875
G	RAD9A	RB1	RBBP8	RBL1	RBL2	SERTAD1	SKP2	STMN1	TFDP1	TFDP2	TP53	WEE1
	NM_004584	NM_000321	NM_002894	NM_002895	NM_005611	NM_013376	NM_005983	NM_005563	NM_007111	NM_006286	NM_000546	NM_003390
H*	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC
	NM_001101	NM_004048	NM_002046	NM_000194	NM_001002	SA_00105	SA_00104	SA_00104	SA_00104	SA_00103	SA_00103	SA_00103

Table 2
gDNA elimination mixture.

Reagent	Volume (µl)
Total RNA	1 µg*
GE (5x gDNA Elimination Buffer)	2
RNase-free water to a final volume	10

* The volume in µl depends on concentration of the RNA in ng/µlThe gDNA mixture was incubated at 42°C for 5min and immediately placed on ice for 1min.

Table 3
Reverse transcription (RT) cocktail.

Reagent	Volume (µl)
- Quantiscript RT Buffer	4
- Quantiscript Reverse Transcriptase	1
- RT Primer Mix	2
RNase-free water	3
Final volume	10

RNA Extraction

RNA was extracted from cells treated for 48h at drug plasma levels 32 µM LPV/r and from control (vehicle-methanol) cells, using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Briefly, cells were washed with PBS twice, and then lysed directly by adding 350 µl of Buffer RLT, to T25 cell culture flasks (for A549 cells) and adding 600 µl of Buffer RLT to T75 cell culture flasks (for MRC-5 cells). Cell scrapers (Lonza) were used to detach the cells, as trypsinisation has been reported to interfere with quality of RNA [19]. The lysate was then transferred into sterile 2ml micro-centrifuge tubes. The tubes were then briefly vortexed before equal volumes (350 µl or 600 µl) of 70% ethanol was added to the lysate. Up to 700 µl of the sample was transferred into an RNeasy Mini spin column placed in a 2ml collection tube. These were centrifuged for 15s at 8000g. The flow-through was then discarded and 700 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged for 15s at 8000g. Once again the flow through was discarded and 500 µl of Buffer RPE was added to the RNeasy spin column, which was centrifuged for 15s at 8000g. The flow through was discarded again, and 500 µl of Buffer RPE was added and the tubes were centrifuged for 2min at 8000g. The flow-through was discarded and the RNeasy spin-column was centrifuged at 8000g for 1min. The RNeasy spin column was inserted into a new 1.5ml collection tube and 30 µl of RNase-free water was added directly to the spin column membrane. This was incubated at room temperature for 1min to allow the RNase free water to cover the spin column membrane. Total RNA was eluted by centrifuging at 8000 x g for 1min. The quality and quantity of RNA was measured using a Nanodrop (Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies)). For highly pure RNA, the 260/280 absorption ratio is ~2, and the 260/230 ratio is 2.0-2.2.

cDNA synthesis

cDNA was synthesized using the RT^2 First Strand cDNA synthesis kit (Qiagen) following the manufacturer's instructions. Briefly, two mixtures were prepared separately, firstly the Genomic DNA (gDNA) elimination mixture (Table 2) for each RNA sample and secondly, the reverse transcription (RT) cocktail (Table 3).

The RT cocktail was briefly centrifuged and added to the gDNA mixture to provide a final volume of 20 µl. This mixture was then incubated at 42°C for 15min, 95°C for 5min and then at 4°C for 5min. cDNA was diluted to 111 µl by adding nuclease-free water, according to the manufacturer's instructions. The cDNA was used immediately for the PCR Arrays or stored at -20°C for future use.

Table 4
The RT-qPCR master mix for PCR Arrays.

Reagent	Volume (µl)
2 × SYBR Green/ROX master mix	1350
cDNA	102
Nuclease-free water	1248

PCR Array plates

The synthesized cDNA was mixed with the Qiagen RT^2 SYBR Green/ROX qPCR master mix, to which nuclease-free water was also added. Thereafter, 25 µl was loaded per well into the 96-well plates which contained pre-dispensed primer sets of the RT^2 Profiler PCR Array, as indicated in Table 4.

The PCR Array plates were centrifuged briefly (Boeco) before they were loaded on the ABI 7500 RT PCR instrument (Applied Biosystems). The following cycling conditions were used: 10min at 95°C, followed by 40 cycles of 15s at 95°C and 1min at 60°C. Data (CT values) was exported as Excel files to (Qiagen) GeneGlobe Database for analysis.

Data Analysis using GeneGlobe

The online GeneGlobe (Qiagen) analysis tool was used to analyse the PCR array data of all treated and untreated samples. A cycle threshold (Ct) of 35 was set as the cut-off value for gene expression. Thus genes with Ct values greater than 35 cycles were considered non-detectable. Five house-keeping genes, including (ACTB-Beta Actin (ACTB); Beta-2-microglobulin (BM2); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); HPRT1-Hypoxanthine phosphoribosyltransferase (HPRT1) and RPLPO-Ribosomal protein, large, P0 (RPLPO)) were used to obtain the change in cycle threshold (ΔCT) value for each gene of interest, where ΔCT is calculated as: CT value of gene of interest less the CT value of reference gene. The difference between the ΔCT of the treatment group and that of the vehicle control group is represented by $\Delta\Delta$CT values, where the fold-change is calculated by $2^{(-\Delta\Delta CT)}$. [20]. This value represents the level of the expression of each gene in the drug-treated (EFV or LPV/r) sample *versus* that in the vehicle control sample. By convention, the fold difference of $\pm$2 up-or-down regulation was used as a basis of target selection. The PCR array results were confirmed by RT-qPCR.

Validation of Cell-cycle Array Data

Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) has been proven to be a more sensitive method for gene expression studies, compared to the conventional method [21]. To validate the PCR Array data, Real-Time quantitative PCR (RT-qPCR) was performed at least three independent times in triplicate. Three representative genes of the array were rationally selected according to their level of expression (above or below two-fold expression).

Primer Design

The Primer 3 tool (http://primer3.wi.mit.edu/) was used to design primers for the selected gene targets, including the house-keeping gene (HKG) GAPDH, based on NCBI accession numbers provided by Qiagen (for all three selected targets), following PCR Array analysis. The target genes selected included MAD2L2 (accession number NM_006341), CASP3 (accession number NM_004346), AURKB (accession number NM_004217) and (HKG) GAPDH (accession number NM_002046). The amplicons' sizes were as follows; 157bp for MAD2L2, 149bp for CASP3, 150bp for AURKB and 87bp for GAPDH. Primer sequences of the selected gene targets and GAPDH as a reference gene (one of the internal controls' panel in the PCR Array) are shown below, Table 5.

Total RNA from LPV/r treated and control cells, treated for 24h and 48h, was extracted using the RNeasy mini kit (Qiagen), as described above. The extracted RNA (treated and control) samples were used for reverse transcription in RT-qPCR experiments. Briefly, RNA was reverse transcribed to

Table 5
Primer sequences of target genes.

MAD2L2	
MAD2L2-Forward:	5′-CGA GTT CCT GGA GGT GGC TGT GCA TC-3′
MAD2L2-Reverse:	5′-CTT GAC GCA GTG CAG CGT GTC CTG GAT A-3′
Caspase 3 (CASP3)	
CASP3- Forward:	5'-GCT CAT ACC TGT GGC TGT GTA-3'
CASP3- Reverse:	5'-ATG AGA ATG GGG GAA GAG GCA -3'
AURKB	
AURKB- Forward:	5'-AGC AGC GAA CAG CCA CG-3'
AURKB- -Reverse:	5'-GCC GAA GTC AGC AAT CTT CA-3'
GAPDH	
GAPDH- Forward:	5'-TGCACCACCAACTGCTTAGC-3'
GAPDH- Reverse:	5'-GGCATGGACTGTGGTCATGAG-3'

cDNA using the Maxima First Strand cDNA synthesis kit for RT-qPCR with dsDNase (Thermo Fischer Scientific), following the manufacturer's instructions as shown in Tables 6 and 7.

The contents were gently mixed and centrifuged. This was followed by incubation of samples at 37°C for 2 min, chilling samples on ice, another brief centrifuge and thenthe samples were placed on ice. Components of Table 2.5 were added to those listed in table 3.5. The two cocktails were gently mixed and briefly centrifuged. The reactions were incubated at 25°C for 10min and 50°C for 15min. The reactions were terminated by heating at 85°C for 5 min. This was followed by 4°C hold for 5min. The cDNA was used immediately Table 8 or was stored at -20°C for future use.

The reactions each consisting of a total volume of 10 µl were then loaded into a 96-well PCR plate, which was centrifuged at 1000rpm for 20s, (Boeco) before loading onto the ABI 7500 RT-qPCR Instrument (ABI,) The following thermal profile was used: denaturation at 95°C for 10min; followed by 40 cycles of 95°C for 15s, then 60°C for 1min (data collection point); and finally the dissociation stage, which is pre-set on the ABI 7500 system at 95°C for 15s, Reps1, 60°C for 1min, 95°C for 15s.

RT-qPCR data analysis

Following the RT-qPCR experimental run on the ABI 7500 light cycler, the CT values were exported as an Excel file to create a table of CT values. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to controls and test groups. CT values were then normalized based on a manual selection of the GAPDH reference gene. The

Table 6
Digestion of gDNA.

Reagent	Volume (µl)
10 × dsDNase Buffer	1
dsDNase	1
Template RNA	1 µg*
Nuclease-free water	Up to 10
Total volume	10

* Added volume of RNA template varies, to amount to 1 µg.

Table 7
Reverse Transcription Components.

Reagent	Volume (µl)
5X reaction mix	4
Maxima enzyme mix	2
Nuclease-free water	4
Total volume	10

Table 8
RT-qPCR master mix cocktail.

Reagent	Volume (µl)
Fwd Primer*	0.4
Rv Primer*	0.4
2X SYBR Green master mix	5
cDNA	0.8#
Nuclease-free water	3.4
Total volume	10

* 10 µM of each primer (for all different gene targets) was used.
# No template reactions were also included, for negative control(s).

data analysis web portal calculates fold change/regulation using the delta delta CT method [20,22] in which delta CT is calculated between genes of interest, (GOI) and HKG, followed by delta-delta CT calculations (delta CT (test/experiment)-delta CT (control/calibrator)). Fold Change is then calculated using 2^ (-delta delta CT) formula, shown below.

$$\Delta CT = CT\left(Test_{target} - Test_{HKG}\right)$$

$$\Delta CT = CT\left(Calibrator_{target} - Calibrator_{HKG}\right)$$
$$= 2^{-[\Delta CT\ (test) - \Delta CT\ (calibrator)]}$$

To understand the pattern of differentially expressed genes, the Ingenuity Pathway Analysis (IPA) bioinformatics tool was used.

Bio-informatics for Pathway analysis

In-silico analyses using various bioinformatics tools provide an important means for analyzing genes, their products and the (disease) pathways they are involved in [23] . The gene-lists represented by the GenBank accession numbers of selected gene targets (from the gene array data) were up-loaded on the IPA database.

Qiagens's Ingenuity Pathway Analysis (IPA) has been widely used to model, analyse and understand the complex biological and chemical systems by the scientific community. In this study, IPA was used to help build a more complete regulatory picture and gain a better understanding of the biology underlying the studied gene expression profiles in response to LPV/r drug treatment. To achieve this, the canonical pathway function and the core analysis function of target genes were used. The GenBank accession numbers of the differentially expressed genes were uploaded on IPA. The representation of canonical pathways was achieved by the use of the z score. The z core was primarily used to indicate the degree of expression levels, with positive z score denoting upregulation, negative z core representing down-regulation, while zero (0) z core illustrate unchanged gene expression. Next, the core analysis function of IPA helps build a biological picture of the observed differential expression patterns.

Results and discussion

DDR is one of the cell's mechanisms to maintain genome integrity following genotoxic insults. Furthermore, gene profiling of DEGs often reveals the underlying molecular mechanisms. The cytotoxic effects of protease inhibitors such as LPV/r have been previously reported [24]. Furthermore, various HAART components' anti-proliferative effects have been previously reported [25]. However, to the best of our knowledge, our study is the first to reveal the upregulated p53 DDR pathway in response to LPV/r treatment in lung adenocarcinoma cells. This was accompanied by loss in nuclear architecture. Examination of genes involved in regulation of the cell cycle revealed how LPV/r treatment induces

the expression of genes that negatively regulate the cell cycle such as the inhibitors of the cyclin dependent kinases (CDKIs), while repressing cell cycle positive regulators. The Aurora transcript variants, Aurora Kinase A (AURKA) and AURKB, reported to be overexpressed in various cancers, were also observed to be upregulated in A549 cancer cells and downregulated by LPV/r treatment [26]. In addition, MAD2L2 was downregulated while CASP3 was upregulated by LPV/r treatment in cancer cells. Profiling of 84 genes related to the cell cycle in response to LPV/r treatment has been insightful, compared to single-gene targeted studies. The IPA bioinformatics tool has also played a significant role in building a biological picture of the observed differential gene expression patterns. Additional mechanistic studies to reveal LPV/r anti-cancer properties will help shed more light on this drug's potential use as an anti-cancer therapeutic.

In clinical settings, patients are exposed to ARV treatment for prolonged periods. Although this study potentially demonstrates the genotoxic effects of LPV/r on normal MRC-5 lung cells, and anti-proliferative effects on A549 adenocarcinoma cells, comparing these *in vitro* findings with *in vivo* results would be beneficial. Future research could include patient sample studies, protein expression level studies of target proteins and microRNA targets regulating the identified pathways.

Conclusions

The research approach employed in this study revealed the genotoxic effect of LPV/r on lung cancer and normal cells. The mechanisms used by this dual protease inhibitor in healthy and cancerous cells are similar, although most of the gene targets in normal cells remain repressed. Further studies are warranted to reveal the severity of LPV/r genotoxicity not only in normal lung cells, but on other types of healthy cells, as this could limit the potential use of LPV/r as an anti-cancer agent. Nonetheless, emerging research reveals the repurposing of antiretroviral drugs as anti-cancer drugs. Despite the cutting-edge diagnostic methods and therapeutics, cancer still remains a leading cuase of death globally. There is therefore an urgent need for improved cancer therapeutic options. Due to time and financial constraints in anti-cancer novel drug development, drug repurposing of the already FDA approved drugs holds promising potential to cancer management, as nelfinavir is a promising PI for cancer drug repurposing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the South African Medical Research Council (SAMRC) for funding.

References

[1] J.A. Barta, C.A. Powell, J.P. Wisnivesky, Global epidemiology of lung cancer, Ann. Glob. Health 85 (1) (2019 Jan 22) 8, doi:10.5334/aogh.2419.
[2] B. Lubuzo, T. Ginindza, K. Hlongwana, The barriers to initiating lung cancer care in low-and middle-income countries, Pan Afr. Med. J. 35 (2020 Feb 12) 38, doi:10.11604/pamj.2020.35.38.17333.
[3] C. Koegelenberg, T. Van Der Made, J Taljaard, E.M. Irusen, The impact of HIV infection on the presentation of lung cancer in South Africa, S. Afr. Med. J. 106 (2016) 666–668, doi:10.7196/SAMJ.2016.v106i7.10737.
[4] R. Visconti, R. Della Monica, D. Grieco, Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword, J. Exp. Clin. Cancer Res. 35 (1) (2016 Sep 27) 153, doi:10.1186/s13046-016-0433-9.
[5] J. Bai, Y. Li, G. Zhang, Cell cycle regulation and anticancer drug discovery, Cancer Biol. Med. 14 (4) (2017 Nov) 348–362, doi:10.20892/j.issn.2095-3941.2017.0033.
[6] D. Alimbetov, S. Askarova, B. Umbayev, T. Davis, D. Kipling, Pharmacological targeting of cell cycle, apoptotic and cell adhesion signaling pathways implicated in chemoresistance of cancer cells, Int. J. Mol. Sci. 19 (6) (2018 Jun 6) 1690, doi:10.3390/ijms19061690.
[7] World Health Organisation. Update of recommendations on first- and second-line antiretroviral regimens. 2019.
[8] J. Borojerdi, N.A. Warfel, E.R. Gardner, M. Danish, M.C. Hollander, S. Kawabata, M. Tsokos, W.D. Figg, P.S. Steeg, P.A. Dennis, Nelfinavir, a lead HIV protease inhibitor, is a broad-spectrum, anticancer agent that induces endoplasmic reticulum stress, autophagy, and apoptosis in vitro and in vivo, Clin. Cancer Res. 13 (17) (2007) 5183–5194.

[9] J.Y. Kim, Y.J. Park, B.M. Lee, S. Yoon, Co-treatment with HIV protease inhibitor nelfinavir greatly increases late-phase apoptosis of drug-resistant KBV20C cancer. (2019).
[10] S. Mortazavi-Derazkola, S. Zinatloo-Ajabshir, M. Salavati-Niasari, Facile hydrothermal and novel preparation of nanostructured Ho2O3 for photodegradation of eriochrome black T dye as water pollutant, Adv. Powder Technol. 28 (3) (2017) 747–754.
[11] S. Zinatloo-Ajabshir, et al., Green synthesis of dysprosium stannate nanoparticles using *Ficus carica* extract as photocatalyst for the degradation of organic pollutants under visible irradiation, Ceram. Int. 46 (5) (2020) 6095–6107.
[12] S. Zinatloo-Ajabshir, Z. Salehi, M. Salavati-Niasari, Green synthesis and characterization of Dy2Ce2O7 nanostructures using Ananas comosus with high visible-light photocatalytic activity of organic contaminants, J. Alloys Compd. 763 (2018) 314–321, doi:10.1016/j.jallcom.2018.05.311.
[13] F. Razi, S. Zinatloo-Ajabshir, M. Salavati-Niasari, Preparation, characterization and photocatalytic properties of Ag2ZnI4/AgI nanocomposites via a new simple hydrothermal approach, J. Mol. Liq. 225 (2017) 645–651, doi:10.1016/j.molliq.2016.11.028.
[14] Z. Zinatloo-Ajabshir, S. Zinatloo-Ajabshir, Preparation and characterization of curcumin niosomal nanoparticles via a simple and eco-friendly route, J. Nanostruct. 9 (4) (2019) 784–790, doi:10.22052/JNS.2019.04.020.
[15] S. Zinatloo Ajabshir, N. Taheri Qazvini, Effect of some synthetic parameters on size and polydispersity index of gelatin nanoparticles cross-linked by CDI/NHS system, J. Nanostruct. 5 (2) (2015) 137–144 https://www.sid.ir/en/journal/ViewPaper.aspx?id=490390.
[16] S. Zinatloo Ajabshir, N. Taheri Qazvini, Inverse miniemulsion method for synthesis of gelatin nanoparticles in presence of CDI/NHS as a non-toxic cross-linking system, J. Nanostruct. 4 (3) (2014) 267–275 https://www.sid.ir/en/journal/ViewPaper.aspx?id=491478.
[17] S. Zinatloo-Ajabshir, M. Baladi, O. Amiri, M. Salavati-Niasari. (2020). Sonochemical synthesis and characterization of silver tungstate nanostructures as visible-light-driven photocatalyst for waste-water treatment, separation and purification technology, 248, 1383-5866, doi:10.1016/j.seppur.2020.117062.
[18] B. Chazotte, Labeling nuclear DNA using DAPI, Cold Spring Harb. Protoc. (2011) pdb. prot5556.
[19] P. Vrtačnik, Š. Kos, S.A. Bustin, J. Marc, B. Ostanek, Influence of trypsinization and alternative procedures for cell preparation before RNA extraction on RNA integrity, Anal. Biochem. 463 (2014) 38–44, doi:10.1016/j.ab.2014.06.017.
[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[21] M. Sanguinetti, et al., Comparison of real-time PCR, conventional PCR, and galactomannan antigen detection by enzyme-linked immunosorbent assay using bronchoalveolar lavage fluid samples from hematology patients for diagnosis of invasive pulmonary aspergillosis, J. Clin. Microbiol. 41 (2003) 3922–3925.
[22] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108.
[23] W. Bechtel, Using computational models to discover and understand mechanisms, Stud. History and Philosohpy Sci. 56 (2016) 113–121.
[24] D. Maksimovic-Ivanic, P. Fagone, J. McCubrey, K. Bendtzen, S. Mijatovic, F. Nicoletti, HIV-protease inhibitors for the treatment of cancer: repositioning HIV protease inhibitors while developing more potent NO-hybridized derivatives? Int. J. Cancer 140 (2017) 1713–1726.
[25] M. Hecht, S. Erber, T. Harrer, H. Klinker, T. Roth, H. Parsch, N. Fiebig, R. Fietkau, L.V. Distel, Efavirenz has the highest anti-proliferative effect of non-nucleoside reverse transcriptase inhibitors against pancreatic cancer cells, PLoS One 10 (6) (2015 Jun 18) e0130277, doi:10.1371/journal.pone.0130277.
[26] A. Tang, K. Gao, L. Chu, R. Zhang, J. Yang, J. Zheng, Aurora kinases: novel therapy targets in cancers, Oncotarget 8 (14) (2017 Apr 4) 23937–23954, doi:10.18632/oncotarget.14893.