Differential Expression of Hub Genes and Activation of p53 by Anti-cancer Compound Curaxin CBL0137

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ABSTRACT
Cancer is a global concern, and there is a need for effective drugs. CBL0137 is a small water-soluble molecule and a new second-generation compound in the family of curaxins with potential anti-cancer activity. Curaxins in general, including CBL0137, intercalate into DNA, act by targeting the histone chaperone 'facilitates chromatin transcription' (FACT) complex, and have the potential to treat tumors by reducing the growth of cancer cells which is shown in a variety of cell lines and animal models. CBL0137 is found to activate the tumor suppressor gene p53. However, the mechanism of p53 activation is poorly understood. Utilizing bioinformatics analysis on available datasets of CBL0137 treated cancer cells of glioma, cervical and multiple myeloma, differentially expressed genes (DEGs) that may lead to the activation of p53 were examined. Three GEO datasets of cells treated with various concentrations of CBL0137 were analyzed, namely HSJD-DIPG007 (GSE153441), MM1.S (GSE117611), and HeLa S3 (GSE117611). The DEGs were identified based on p-values less than 0.05, logFC values greater than 1 and less than -1 and analyzed using GEO2R, Enrichr, and STRING, and data visualization was performed on Tableau. Compared to the controls, a total of 229, 1425, and 1005 genes were upregulated while 368, 2322, and 1673 genes were downregulated for HSJD-DIPG007, MM1.S, and HeLa S3 datasets, respectively. Further collective analysis revealed a total of 38 common DEGs among the three datasets. Using Enrichr and STRING on these 38 DEGs, seven hub genes were obtained, SKP2, RGS16, CSRP2, CENPA, HJURP, DTL, and HEXIM1 with these possible mechanisms: inhibition of AKT phosphorylation by upregulated genes RGS16 and CSRP2, p300-mediated acetylation of p53 via SKP2, inhibition of MDM2 by DTL downregulation and HEXIM1 upregulation, and inhibition of AURKB via CENPA and HJURP downregulation. This study analyzed the three datasets and highlighted how these identified hub genes might play a role in leading to p53 activation by CBL0137.

KEYWORDS
Curaxin; CBL0137; Differentially Expressed Genes; Cancer; p53; Glioblastoma; Cervical; Myeloma

INTRODUCTION

In 2022, it is estimated that there will be 1.9 million new cancer cases with 609,360 cancer-related deaths in the United States alone.[1] There are a plethora of intolerable side effects of chemotherapy, and additionally, the clinical efficacy of chemotherapy can be compromised due to the possibility of drug resistance during treatment. New agents are being discovered or synthesized and investigated for this reason. Through research and experimentation, the anti-malarial compounds known as curaxins were developed in their second-generation group, of which the member CBL0137 has been deemed an agent with anticancer activity and examined Phase I/II trial.[2] According to literary findings, curaxin has displayed antitumor activity in glioblastoma, renal cell carcinoma, melanoma, neuroblastoma, and small cell lung cancer.[3]

CBL0137 exerts tumor suppression activity in part by activating the tumor suppressor gene p53.[3-5] However, the mechanisms through which this occurs are not completely understood. Systematically, CBL0137 is identified to act via the inhibition of the facilitating chromatin transcription (FACT) complex which is composed of two subunits, SPT16 and SSRP1, and is responsible for the disassembly and reassembly of nucleosomes mandatory for transcription, DNA replication, and repair.[3-5] SSRP1 affects p53 activity via association with the kinase CK2, leading to activation of p53 and inhibition of NF-kB.[3] It is not known if CBL0137 affects other well-known mechanisms that activate p53, such as AKT phosphorylation, p-300 mediated acetylation signaling pathway, p53/MDM2 pathway, and AURKB inhibition.[6-9] The objective of this research study is to analyze CBL0137's effects on gene expression leading to p53 activation in cancer cells by using expression profile datasets of treated and untreated glioma cells, and cervical and multiple myeloma cell lines.

METHODS AND PROCEDURES

GEO Datasets

Pubmed was used as a biomedical/life science search engine and database to conduct research on Curaxin CBL0137, the divergent pathways through which it may function, and the various genes that play a significant role in the mechanisms. GEO DataSets (https://www.ncbi.nlm.nih.gov/gds) was used to find datasets with CBL0137 treatment. The expression profiling by array from three cell types: HSJD-DIPG007 diffuse intrinsic pontine glioma cells (GSE153441), HeLa S3 cervical cancer cell line (GSE117611), and MM1.S multiple myeloma cell line (GSE117611) that consisted of untreated or treated with concentrations of 0.6 μM, 3 μM, and 3 μM of CBL0137, respectively, were used. GSE153441 was provided by Wilkins Lab from The University of New South Wales. They treated HSJD-DIPG007 cells with a single concentration of 0.6 μM of CBL0137. GSE117611 was provided by the Cell Stress Biology Department at Roswell Park Cancer Institute. They treated the MM1.S cell line with 0, 0.3, 1, and 3 μM of CBL0137. They also treated the HeLa cell line with 0 and 3 μM of CBL0137. In this study, we selected the highest CBL0137 concentration available from each dataset because these concentrations were optimal for inhibiting cancer cell growth in their initial studies.[10,11] GEO2R Analyzer (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to compare the control group (no treatment of CBL0137) with test groups (various concentrations of CBL0137) from the same dataset.

Screening Differentially Expressed Genes

Volcano plots were generated with GEO2R Analyzer, Tableau 2020 and *https://paolo.shinyapps.io/ShinyVolcanoPlot/*, for reproducibility and confirmation. We identified genes with -log(p-value) greater than 1.3 and logFC greater than 1 or less than -1. For the volcano plot, all 20,000 to 50,000 genes from all three datasets - obtained from GEO2R Analyzer - were used to identify differentially expressed genes (DEGs). We further determined the common genes between three different datasets and created heat maps to visually identify the differences in fold change across all three datasets.

Enrichment Analysis

We used Enrichr (https://maayanlab.cloud/Enrichr/), an enrichment analysis web-based tool that checks whether an input set of genes significantly overlaps with the annotated gene sets from the database. Enrichr contains 35 set libraries separated into six categories: transcription, pathways, ontologies, diseases/drugs, cell types, and miscellaneous. Once all of the total upregulated and downregulated DEGs were narrowed down to 38 commonly expressed DEGs between the three datasets, the 38 genes were input to Enrichr to compare them against numerous genes of known biological function. In observing the top five pathways of upregulated DEGs and downregulated DEGs in BioPlanet, GO Cellular Component, and KEGG Human, we were able to further analyze specific genes and their biological relevance upon CBL0137 treatment in cancerous cells.

Protein-protein Interaction Networks

STRING (http://www.string-db.org/) is a biological database and online web resource tool of known and predicted direct and indirect protein-protein interactions (PPI's) in numerous, varying organisms. STRING was used to visualize the PPIs between the final list of the 38 statistically significant differentially expressed genes common to all three datasets and the 4 significant differentially expressed genes found via literature evidence that were observed to be common to only two of the three datasets, using Homo sapiens as the model organism. To start off, we input a custom value of 0.5 confidence - the probability of an association between two proteins existing within the same Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway - for the interaction score. This value met the needs of our study as it was in the range of medium confidence (0.4) to high confidence (0.9), highlighting significant protein-protein interactions and producing utilizable data. Each individual PPI is scored on a scale of 0 to 1. This value is known as the combined score; it does not represent the strength or specificity of the association, but rather how likely it is that the interaction is accurate, based on genomic context predictions, high through-put laboratory experiments, conserved co-expressions, automated text mining, and previous knowledge in databases (http://www.string-db.org/).

RESULTS

Identification of 38 Common Differentially Expressed Genes from Three Datasets

Three different datasets representing three cancers and effects by CBL0137 were evaluated. Genes with -log(p-value) greater than 1.3 (means $p < 0.05$) and logFC greater than 1 or less than -1 from HSJD-DIPG007, HeLa S3, and MM1.S datasets were identified as differentially expressed genes (DEGs) from the excel sheet obtained from GEO2R and visualized from the volcano plots. GEO2R's default setting of Benjamini & Hochberg false discovery rate method was applied to the p-values. DEGs are represented by red dots in **Figure 1**. We first identified the total differentially expressed genes in each dataset and then genes common to all three datasets and any two of the datasets, as shown in **Figure 2**. There were 597, 3747, 2678, DEGs for HSJD-DIPG007-CBL0137, MM1.S-CBL0137, and HeLa S3-CBL0137, respectively. Of these, there were 229, 1425, 1005 upregulated genes and 368, 2322, 1673 downregulated genes for HSJD-DIPG007-CBL0137, MM1.S-CBL0137, and HeLa S3-CBL0137, respectively. Downregulated genes are marked in red in the upper-left corner of each graph, whereas upregulated genes are marked in red in the upper-right corner of the graph. It is of interest to note that there were more downregulated genes in all three datasets. Examining the common DEGs in all three cancer datasets, 38 genes were common to all datasets, 51 genes

common to HSJD-DIPG007 and HeLa S3, 73 genes common to HSJD-DIPG007 and MM1.S, and 954 genes common to HeLa S3 and MM1. After analyzing all three datasets together, it is of interest to note that 38 genes out of a total of 65,537 genes were identified as common DEGs.

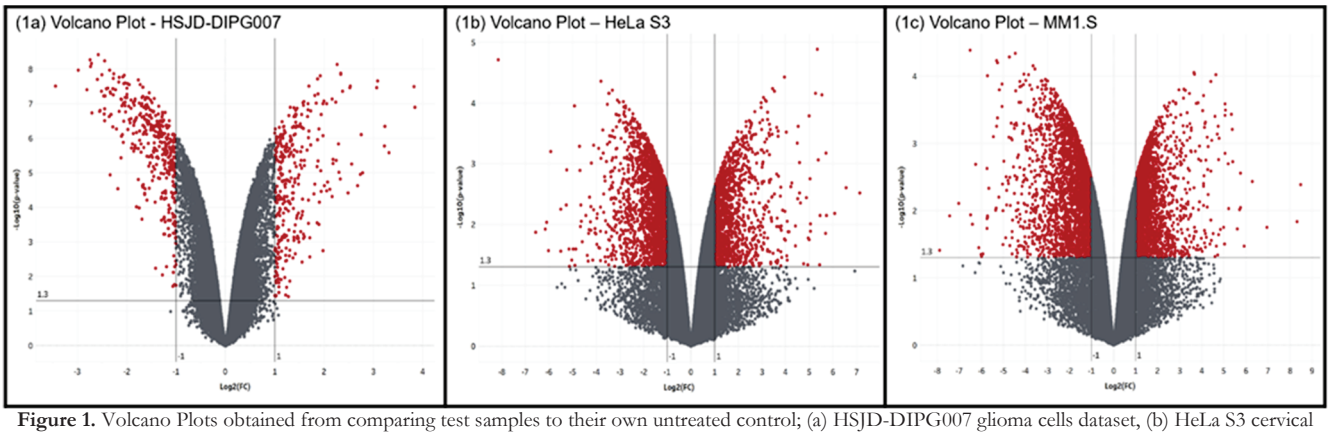

Figure 1. Volcano Plots obtained from comparing test samples to their own untreated control; (a) HSJD-DIPG007 glioma cells dataset, (b) HeLa S3 cervical cancer cell line dataset, and (c) MM1.S multiple myeloma cell line dataset. Upregulated genes are in the upper-right section of the graph and represent logFC > 1 and p-value < 0.05. Downregulated genes are in the upper-left section of the graph and represent logFC < -1 and p-value < 0.05.

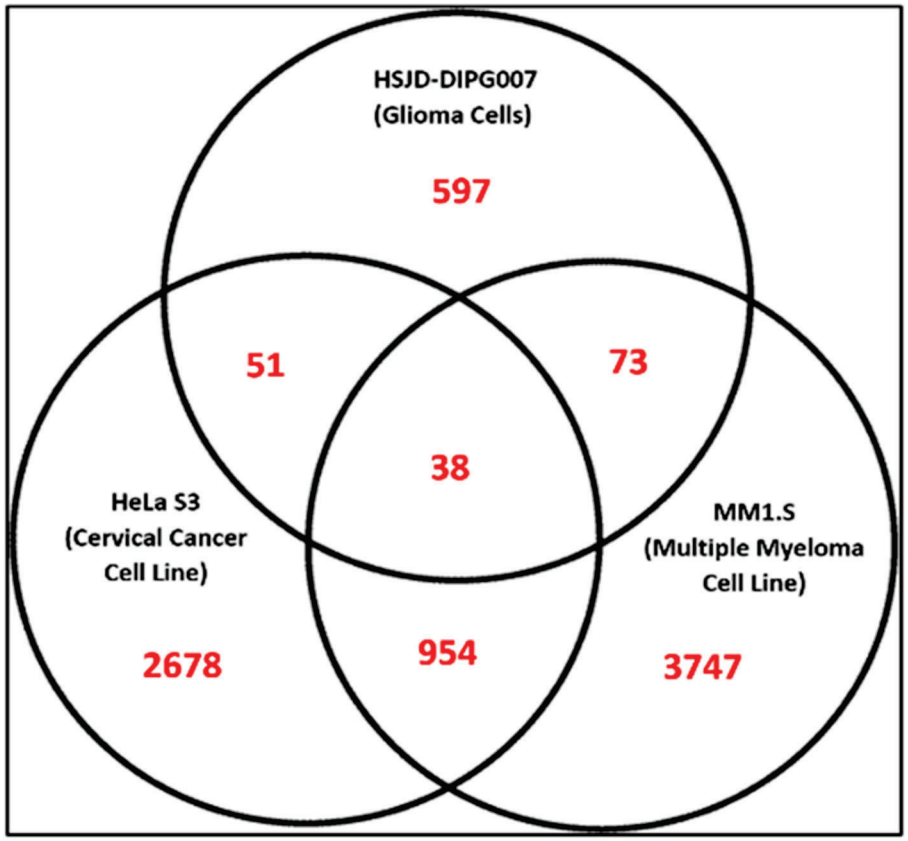

Figure 2. Venn Diagram showing total DEGs in each of the three datasets and overlapping DEGs in two or three of the datasets.

Collation of Datasets Using Heat Map

A heat map was created comparing all three datasets: HSJD-DIPG007 (0.6 μM CBL0137), HeLa S3 (3 μM CBL0137), and MM1.S (3 μM CBL0137). **Figure 3** depicts the 38 common genes in rows, 3 different datasets in columns and color gradient representing logFC of the gene after the treatment of CBL0137 when compared to the control. The majority of the common genes were downregulated in the HSJD-DIPG007, HeLa S3, and MM1.S. On the other hand, AURKA, EPM2AIP1, FAM83D, IFIT2, NBEA, and PRR11 were differentially expressed in different directions in at least one of the datasets.

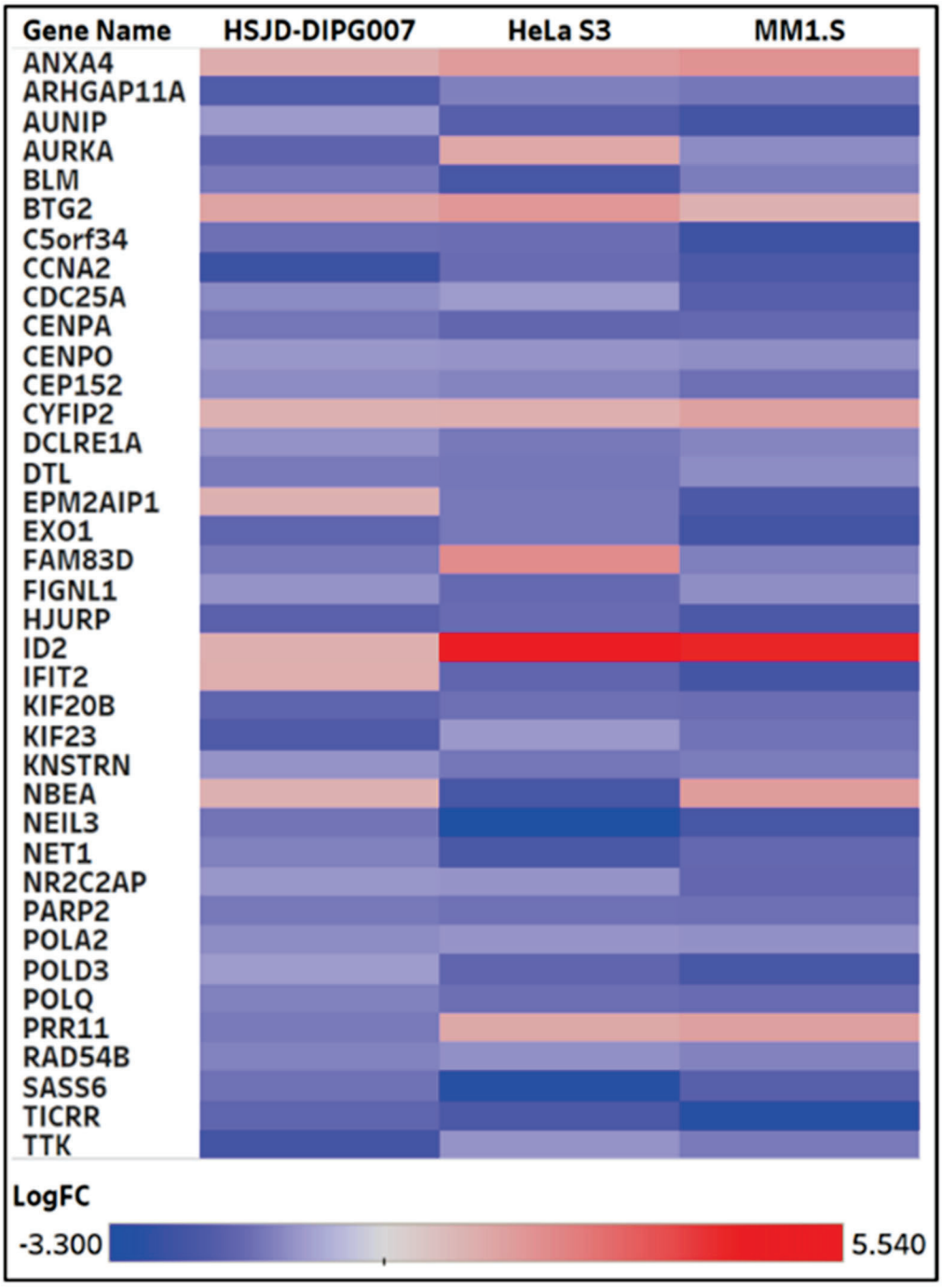

Figure 3. Heat map of the 38 common genes, between three datasets, treated with CBL0137. Red represents upregulated genes, and blue represents downregulated genes. All DEG's have a p-value of less than 0.05.

Relevant Pathways of Upregulated and Downregulated DEGs

Enrichr was used to determine the most relevant five pathways of upregulated DEGs and downregulated DEGs in BioPlanet, GO Cellular Component, and KEGG Human given the 38 common DEGs, as shown in **Figure 4**. In the Enrichr analysis the colored bar graphs represent the significant p-values, and the asterisk next to the p-value corresponds to it having a significant adjusted p-value which was calculated based on the Benjamini-Hochberg method. BioPlanet 2019 showed that most of the 38 common genes are part of the cell cycle, chromosome maintenance, DNA replication, homologous recombination, and base excision repair pathways. The top five pathways in GO Cellular Component 2021 of the 38 common genes are intracellular non-membrane-bounded organelle, spindle, deuterostome, nucleus, and microtubule cytoskeleton. KEGG Human 2019 showed that

most of the 38 common genes are part of the base excision repair pathways, homologous recombination, mismatch repair, progesterone-mediated oocyte maturation and cell cycle.

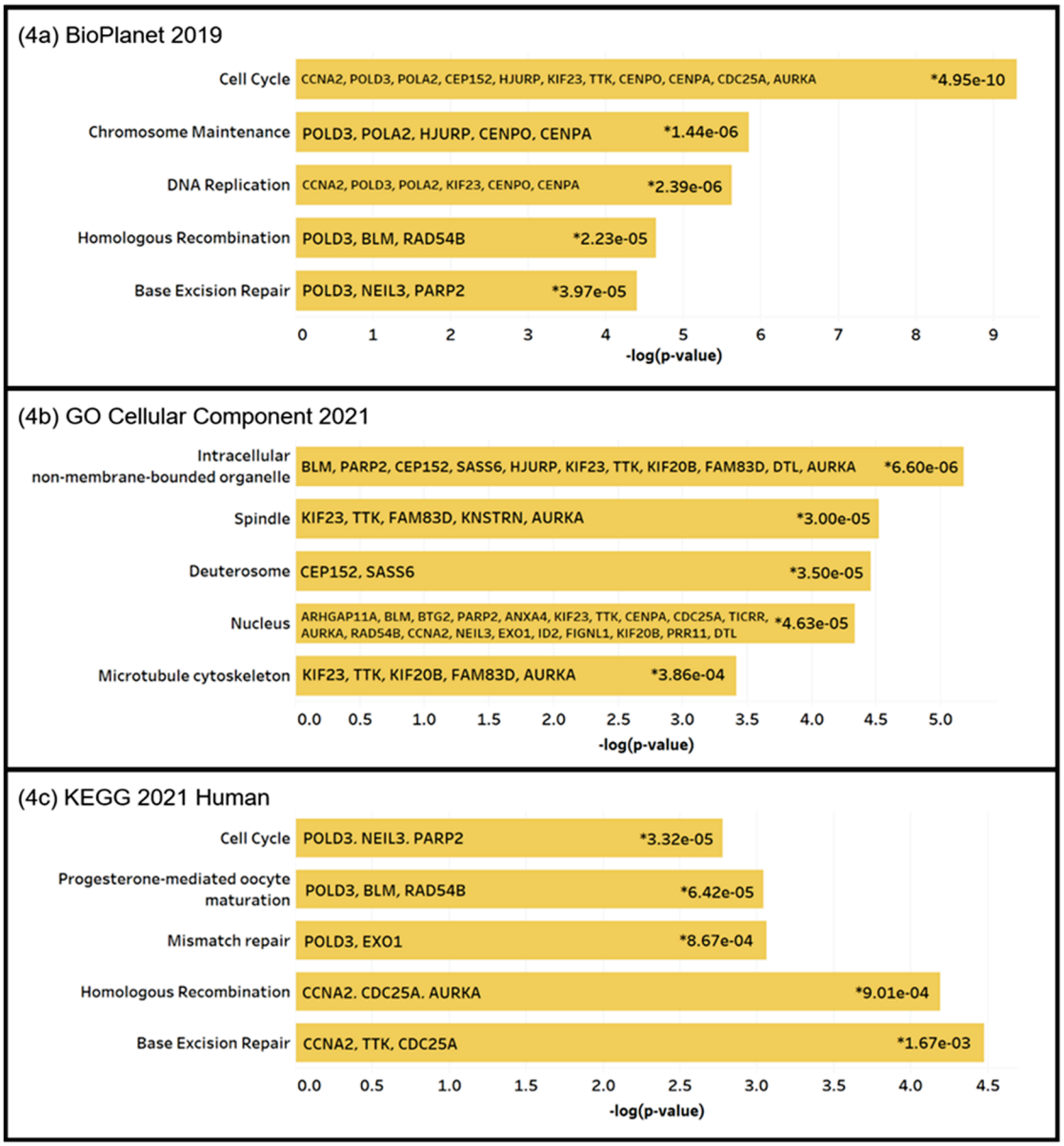

Figure 4. Top five pathways of 38 common DEGs from three datasets in (a) BioPlanet 2019, (b) GO Cellular Component 2021 and (c) KEGG 2021 Human. P-values are shown with *. Corresponding genes of each pathway are listed within the bars of the graphs.

Identification of Hub Genes

STRING, a database of known and conjectured direct and indirect protein-protein interactions, was utilized to depict the protein associations between the 38 common genes, selected upregulated and downregulated genes that were found in only two datasets (RGS16, CSRP2, HEXIM1, and SKP2), and the genes that are already known to act as regulators for the potential candidates: TP53, NFKB1, AKT1, EP300, MDM2, and AURKB. The STRING plot with an interaction score of 0.5, as shown in **Figure 5**, was chosen to be the main focal point as it illustrated all the possible protein-protein interactions that were statistically significant. Certain hub genes were then picked from the STRING plot that were found to have significant effects on p53. The likelihood of the interactions - between certain genes and p53 - to be deemed true was based on the combination score values listed in **Table 1**. Subsequently, all of the 954 differentially expressed genes that were common to the HeLa S3 and MM1.S datasets, such as RGS16 and CSRP2 among 25 genes, were further analyzed on STRING with an interaction score of 0.5 and 0.4 as shown in **Figure 5**. RGS16 and CSRP2 are found to have edges or interaction score of 0.5.

Gene From Dataset	Upregulated or Downregulated	Interaction Score	Interaction with p53 on STRING Plot	Combination Score of Gene with p53 directly	Interaction with a p53 Regulator	Combination Score of Gene with p53 Regulator
RGS16	Upregulated	0.2	Direct and Indirect	0.259	AKT1	0.324
CSRP2	Upregulated	0.2	Direct	0.22	N/A	N/A
CENPA	Downregulated	0.5	Indirect	N/A	AURKB	0.998
HJURP	Downregulated	0.5	Indirect	N/A	AURKB	0.919
TTK	Downregulated	0.5	Direct and Indirect	0.765	AURKB	0.98
KIF23	Downregulated	0.5	Direct and Indirect	0.574	AURKB	0.998
SKP2	Downregulated	0.5	Direct and Indirect	0.971	MDM2 / AKT1 / AURKB / EP300	0.579 / 0.792 / 0.745 / 0.960
DTL	Downregulated	0.5	Direct and Indirect	0.624	MDM2 / AURKB	0.556 / 0.856
HEXIM1	Upregulated	0.5	Direct and Indirect	0.501	MDM2 / AKT1	0.689 / 0.550

Table 1. Interactions and combination score of genes from STRING plot.

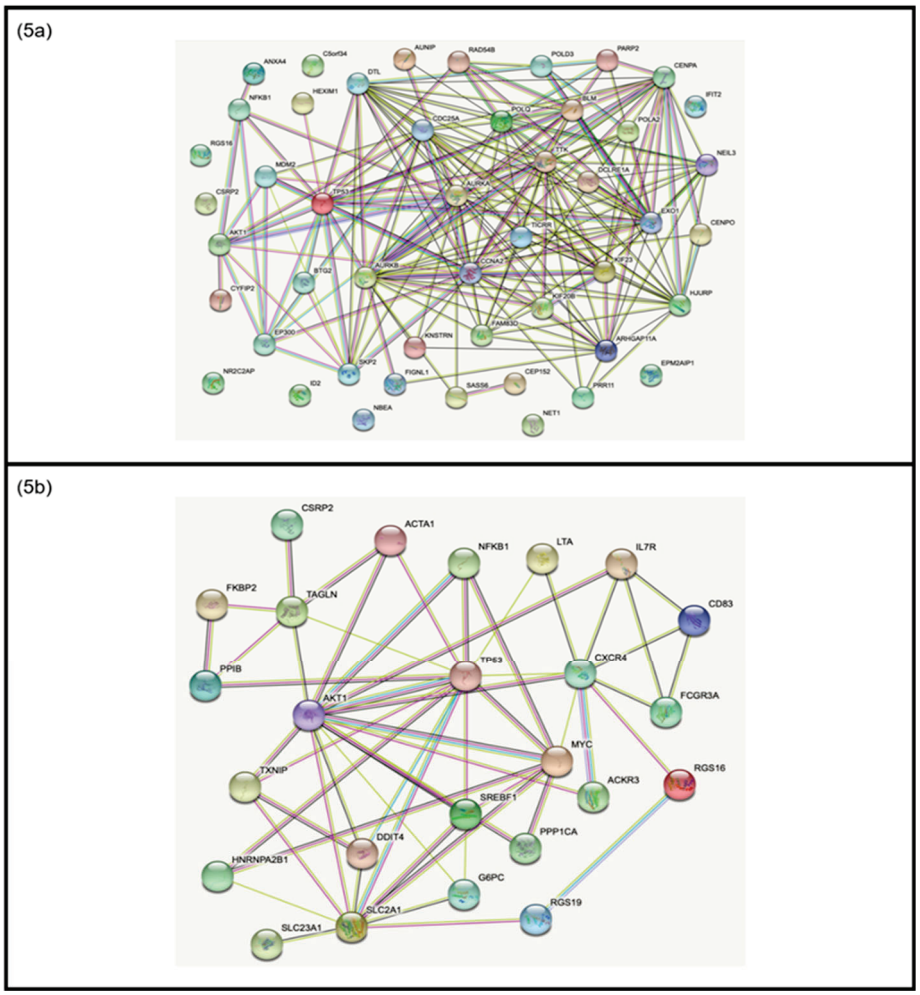

Figure 5. STRING plot depicting protein-protein interactions between the 38 common genes, four selected genes (RGS16, CSRP2, HEXIM1, and SKP2), potential regulators of the tumor suppressor gene (NFKB1, AKT1, EP300, MDM2, and AURKB) and TP53: (a) with an interaction score of 0.5 and (b) with an interaction score of 0.4.

DISCUSSION

Enrichr and String Analysis

This study identified seven potential hub genes – SKP2, RGS16, CSRP2, CENPA, HJURP, HEXIM1, and DTL – that indirectly cause p53 activation and result in tumor suppression, as shown in **Table 2** and **Figure 6**. The BioPlanet 2019 analysis conducted on Enrichr revealed that CENPA and HJURP are part of the cell cycle and chromosome maintenance pathways. Furthermore, it

was found that CENPA is also involved in the DNA replication pathway. GO Cellular Component 2021 showed that HJURP is part of an intracellular non-membrane bound organelle whereas CENPA and DTL are part of the nucleus. STRING plot, created with an interaction score of 0.5, identified five hub genes – SKP2, CENPA, HJURP, DTL, and HEXIM1 – which exhibited the truest protein-protein interactions. Potential associations between p53/RGS16 and p53/CSRP2 were recognized, as seen on the STRING plot with an interaction score of 0.4 and 0.2, and in **Table 1**. The absence of connections involving these two hub genes on the STRING plot with an interaction score of 0.5 are due to the confidence scores which are based on literature evidence. According to several literature sources on breast, colorectal, and pancreatic cancer, it is evident that the genes RGS16, CSRP2, HEXIM1, and SKP2 - while only common to two of the three datasets - are all significant in relation to the activity of the p53 either through a direct relationship or indirectly through its regulators, and therefore are reported in this study.[7,12,14,17]

Genes From Combined Datasets	Combined Datasets	LogFC	Up- or Downregulated Gene	p53 Regulator	p53
SKP2	Glioma	-1.28	Downregulated	p300 binding to SKP2 inhibited	Activation
	HeLa S3	-1.13			
		-1.24			
		-1.78			
RGS16	Hela S3	3.68	Upregulated	AKT phosphorylation inhibited	
	MM1	2.29			
CSRP2	Hela S3	3.52	Upregulated	AKT phosphorylation inhibited	
	MM1	1.73			
CENPA	Glioma	-1.51	Downregulated	AURKB decreases	
	Hela S3	-1.78			
	MM1	-1.73			
HJURP	Glioma	-1.93	Downregulated	AURKB decreases	
	Hela S3	-1.67			
	MM1	-2.22			
DTL	Glioma	-1.46	Downregulated	MDM2 decreases	
	Hela S3	-1.5			
	MM1	-1.19			
HEXIM1	Hela S3	3.17	Upregulated	HDM2 decreases	
	MM1	2.12			

Table 2. Expression of seven hub genes by CBL0137 from the datasets combined and their interaction with regulators that may activate p53.

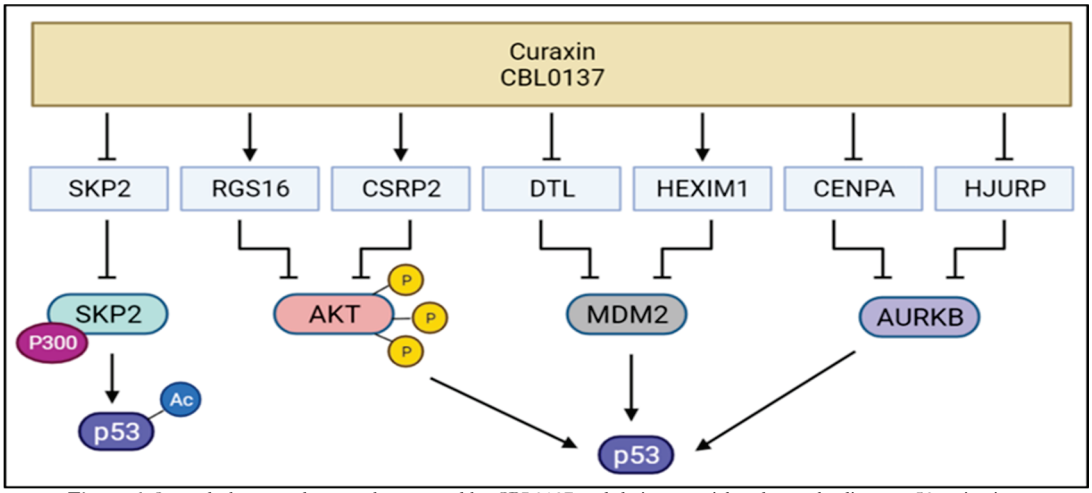

Figure 6. Seven hub genes that may be targeted by CBL0137 and their potential pathways leading to p53 activation.

Inhibition of AKT Phosphorylation by Upregulated Genes RGS16 and CSRP2

RGS16 was found to be upregulated with a logFC of 3.68 and 2.29 in HeLa S3 and MM1.S cell lines, respectively. It has been reported that RGS16 plays a significant role in inhibiting pancreatic cell and breast cancer cell growth.[12,13] Our experimental findings confirm the upregulation of RGS16 by CBL0137 in a cervical cancer cell line, HeLa S3. CSRP2 is another upregulated gene, with a logFC of 3.52 and 1.73 in HeLa S3 and MM1.S cell lines, respectively. Prior studies have shown that CSRP2

promotion is known to suppress colorectal cancer and inhibition of CSRP2 is known to proliferate leukemia cells.[14,15] This study identified and confirmed that both RGS16 and CSRP2 are genes upstream of the AKT signaling pathway and inhibit AKT phosphorylation, resulting in the stabilization of p53.

p53 Acetylation and its Stabilization Via SKP2

SKP2 is a downregulated gene in the glioma and HeLa S3 dataset, with logFC values of -1.28 and -1.24, respectively. CBL0137 inhibits p300 from binding to SKP2, making p300 available to acetylate p53. Acetylation of p53 leads to death of cancer cells. Literature evidence shows that SKP2-mediated inhibition of p300 is known to decrease p53 in breast cancer cells.[7]

Inhibition of HDM2 / MDM2 by DTL Downregulation and HEXIM1 Upregulation

DTL, also known as L2DTL, is a downregulated gene found in the HSJD-DIPG007 cells, MM1.S cell line, and HeLa S3 cell line with logFC values of -1.46, -1.19, and -1.5, respectively. According to scientific literature, DTL has a positive relationship with MDM2; the downregulation of DTL inhibits the proto-oncogene MDM2.[16] Our study confirms the downregulation of DTL by CBL0137, which leads to the regulation of p53 polyubiquitination and induction of p53 expression. HEXIM1 is an upregulated gene found in the HeLa S3 cell line and MM1.S cell line with logFC values of 3.17 and 2.12, respectively. HDM2, a human homolog of MDM2, is known to bind with p53 causing p53 degradation. The binding of HEXIM1 with HDM2 prevents HDM2 from binding with p53 leading to inhibition of p53 ubiquitination.[17]

Inhibition of AURKB by Downregulation of CENPA and HJURP

CENPA is a downregulated gene found in the CBL0137 treated groups of HSJD-DIPG007 cells, MM1.S cell line, and HeLa S3 cell line, with logFC values of -1.51, -1.73, and -1.78, respectively. Scientific research and literary findings have reported that the downregulation of CENPA has significant effects in inhibiting cancer cell growth. It is able to decrease the expression of proto-oncogene AURKB since Aurora B kinase targets CENPA which in turn would result in p53 activation.[9, 18-20] Our experimental findings and statistics confirm the downregulation of CENPA by CBL0137 in all three cell lines analyzed. HJURP is another downregulated gene found in HSJD-DIPG007, MM1.S, and HeLa S3 cell lines. HJURP presented logFC values of -1.93 in Glioma, -2.22 in MM1.S, and -1.67 in HeLa S3. The inhibition of HJURP results in the knockdown of AURKB hence, demonstrating a positive relationship between both genes.[18] Indeed, there is a negative correlation between HJURP and p53 as the inhibition of AURKB leads to senescence by p53 expressing its tumor-suppressing activity.[21] Our study reveals that CENPA and HJURP are both genes upstream of the AURKB pathway, by the effects of CBL0137, and thus lead to p53 activation.

Current Limitations and Future Work

It should be noted that in this study only three cancer cell types were examined. Also, since we were comparing three extremely diverse cancers that are a product of various specific mutations, the response to the treatment with the same anti-cancer drug differed and made some cells more or less insensitive. The datasets used concentrations of CBL0137 appropriate to IC_{50} in the cell type. Further studies include supporting this bioinformatics research with experimental data which can confirm the role of our identified hub genes and proteins leading to p53 activation. In addition, treatment of other cancer cell lines with CBL0137 and examination of elements from pathways described may provide validation. This study highlighted certain critical pathways through which p53 can be activated. One of the most promising genes that should be studied further is CSRP2. Based on our survey of literature of these identified hub genes, CSRP2 and its mechanism of action leading to p53 activation was found to be the least studied. Our findings suggest that the upregulation of CSRP2 can lead to the inhibition of AKT phosphorylation and the detailed mechanisms should be explored further through in vitro studies with MM1.S, HeLa S3, and glioma cells aimed at examining the phosphorylation changes.

CONCLUSIONS

CBL0137 holds promise against various cancers. Using three different datasets, we identified the common differentially expressed genes and selected seven hub genes - RGS16, CSRP2, SKP2, DTL, HEXIM1, CENPA, and HJURP - which play a significant role in three divergent pathways all leading to, and resulting in, p53 stabilization and activation.

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PRESS SUMMARY

This research analyzed common differentially expressed genes by a curaxin member, CBL0137, a potential anti-tumor compound, in datasets of glioblastoma, cervical cancer, and multiple myeloma for potential pathways leading to the activation of p53. Seven hub genes were identified – SKP2, RGS16, CSRP2, CENPA, HJURP, DTL, and HEXIM1 – which may act *via* inhibition of AKT phosphorylation by upregulated genes RGS16 and CSRP2, p300-mediated acetylation of p53 via SKP2, inhibition of MDM2 by DTL downregulation and HEXIM1 upregulation, and inhibition of AURKB via CENPA and HJURP downregulation. Future validation studies for p53 modulation may focus on these genes.