Novel Small-molecule Mdm2 Inhibitor As A Potential Anticancer Agent For Gastric And Breast Cancer

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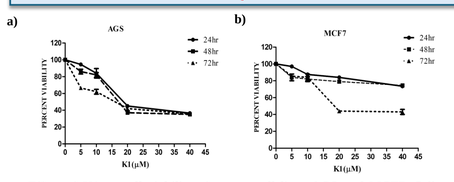
ABSTRACT

Breast and gastric cancers are among the most common cancers worldwide and are strongly influenced by the p53–MDM2 axis, yet clinical development of MDM2 inhibitors has been limited by drug resistance and poor efficacy. We investigated K1, a quinazoline derivative, which demonstrated potent anticancer activity with an IC50 value of 18.5μM in AGS and 19μM in MCF7 cell lines post 72h treatment, and it also significantly reduced their clonogenic potential. Transcriptomic data show that K1 targets multiple stress pathways in distinct cellular compartments. In MCF7, ribosomal stress genes (RPL11, RPL23, RPS7, RPL26) and ER stress markers (DDIT3, ATF4, EIF2AK3) were upregulated. In addition, the expression of apoptotic protein mediators (PMAIP1, BBC3) and DNA damage regulators (CDKN1A, CDKN2D, FHIT) was also upregulated. Whereas in AGS cells, mitochondrial dysfunction and mitophagy indicators (PINK1, PRDX3, PARK7), oxidative stress genes (CYP1A1, BACH1) and apoptotic regulators (FOXO3, RHOB, STAT1) showed increased expression. Elevated expression of mitotic stress and spindle assembly checkpoint genes (PLK1, CDC20, BUB1, MAD2L) leads to G2/M arrest and spindle disruption. This compartment-specific activity indicates a spatial biology perspective, linking gene expression changes to localized stress within the tumor environment. K1 causes nuclear fragmentation, cytoskeletal disruption, ROS generation, mitochondrial dysfunction, and DNA damage. In vitro studies indicated decreased MDM2 with increased p53 and p21 expression in MCF7, while AGS showed a decline in MDM2 and p53, with increased p21 expression. Despite the differences in response, both cell lines ultimately culminated in apoptosis. Molecular docking of K1 with MDM2 exhibited key molecular interactions, binding conformation and stable dynamics. Our data identifies K1 as a promising anticancer candidate that activates compartment-specific, spatially distinct stress response leading to apoptosis in two distinct cancer types.

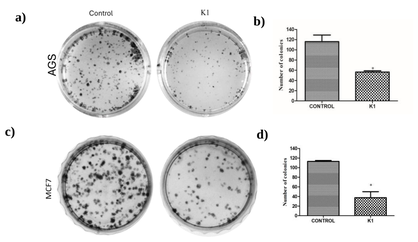
RESULTS

K1 inhibits the viability of AGS and MCF7 cells



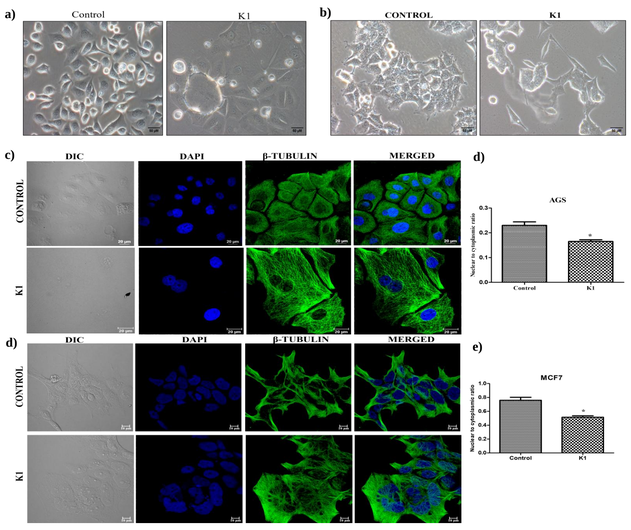
Effect of K1 on cell viability of cancer cell lines AGS and MCF7. Cells were treated with increasing concentrations of K1 for 24,48 and 72hr. Dose-dependent decrease in viability of (a) AGS and (b) MCF7 cells is observed in the presence of indicated K1 concentrations.

K1 inhibits colony formation of cells



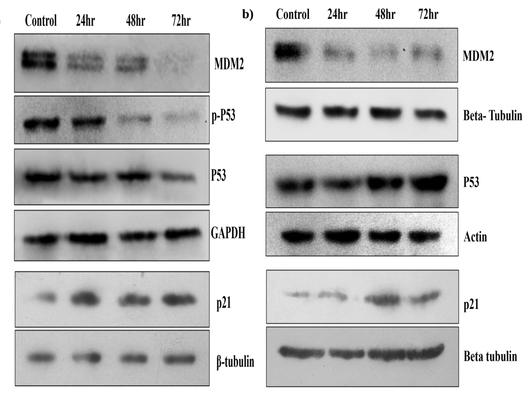
K1 reduced the colony forming ability of AGS and MCF7 cells. (a) AGS and (c) MCF7 cells were treated with K1 for 72hr and then replated by counting 500 cells in a drug free medium. Bar graphs showing decrease in the number of colonies for AGS (b) and MCF7 (d) cells treated with K1 (20μM).

K1 induced morphological changes in cells



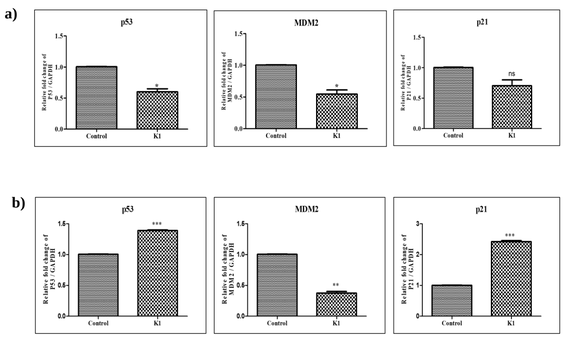
Change in morphology of AGS and MCF7 cells upon K1 treatment. (a) Microscopic images of AGS cells showing enlarged and vacuolated cells upon K1 (20μM) treatment for 72hr. (c) β- tubulin and DAPI staining of AGS cells show the enlarged cytoplasmic area of AGS cells after K1 treatment. (d) Bar graph represents the reduction in nuclear to cytoplasmic ratio of K1 treated AGS cells. (b) Microscopy images of MCF7 cells shows irregular and enlarged cells following K1 (20μM) treatment for 72hr. (d) Confocal fluorescence microscopy images of β-tubulin and DAPI staining of MCF7 cells show the enlarged cytoplasmic area of MCF7 cells and nuclear fragmentation after K1 treatment. (e) Bar graph represents the reduction in nuclear to cytoplasmic ratio of K1 treated cells. P value: *P< 0.05 as compared to respective DMSO treated controls.

Effect of K1 on P53-MDM2 expression



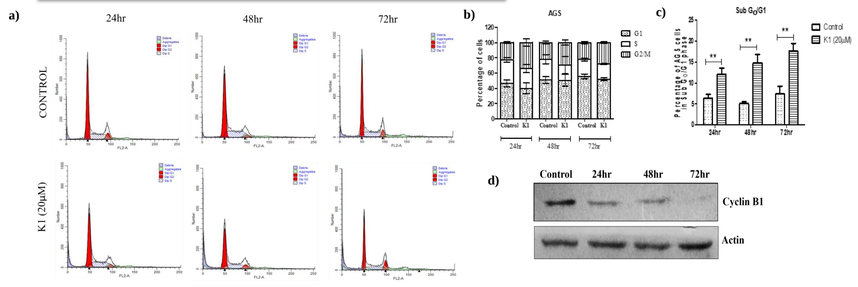
Role of K1 in the regulation of MDM2, p53 and p21 proteins on AGS and MCF7 cells. (a) MDM2, phosphorylated and total p53 protein expression levels significantly decreased in AGS cells upon K1 (20μM) treatment, whereas an increase in p21 expression level was observed. (b) A decrease in MDM2 protein level, and an increase in p53 and p21 protein expression levels was observed in MCF7 cells upon K1 (20μM) treatment. Values on the western blots denote their relative intensity.

K1 regulates mRNA expression in AGS and MCF7

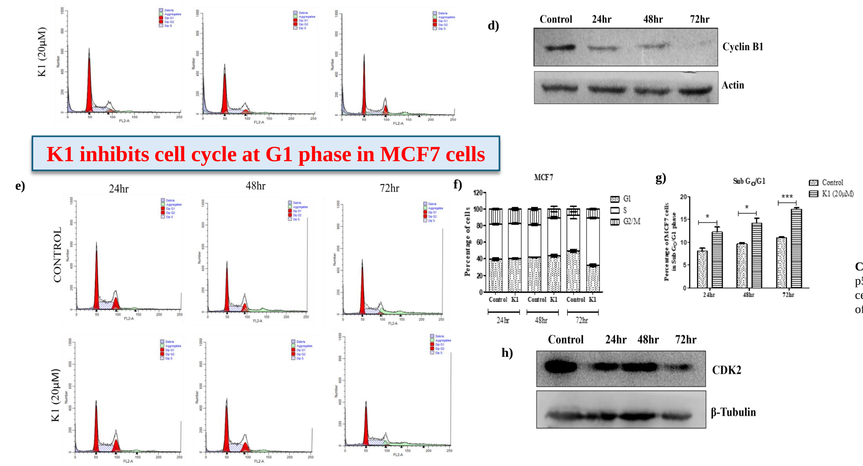


Role of K1 in the regulation of MDM2, p53 and p21 mRNA levels in AGS and MCF7 cells. (a) mRNA expression of p53 , MDM2 and p21 in AGS cells and (b) mRNA levels of p53 , MDM2 and p21 in MCF7 cells upon K1 (20μM) treatment.

K1 inhibits cell cycle at G2/M phase in AGS cells

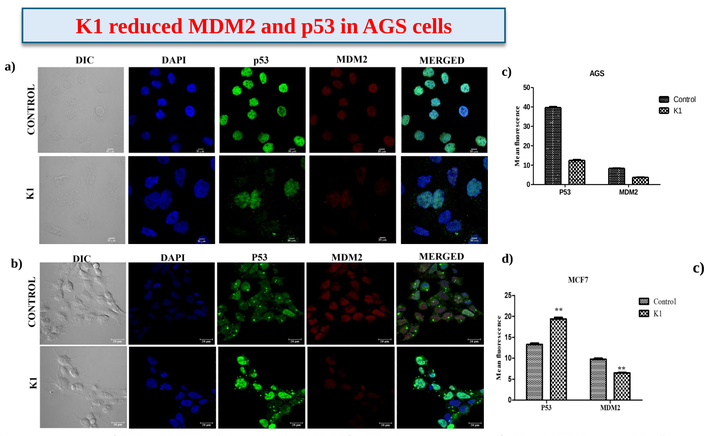


K1 inhibits cell cycle at G1 phase in MCF7 cells



K1 induces cell cycle arrest in AGS and MCF7 cells. Histograms depicting cell cycle phases at different time points of 24, 48 and 72hr upon K1 (20μM) treatment on AGS (a) and MCF7 cells (e). Bar graphs showing the percentage of cells in G1, S and G2/M phases of the cell cycle in (b) AGS and (f) MCF7 cells with K1 treatment at 24, 48 and 72hr. Accumulation of cells at Sub G_0G_1 phase upon K1 treatment in AGS (c) and MCF7 (g). Decrease in cyclin B1 expression in AGS cells (d) and decreased CDK2 in MCF7 (h). P value: *P< 0.05, **p< 0.01 and ***P< 0.001 as compared to respective DMSO treated controls.

K1 reduced MDM2 and p53 in AGS cells

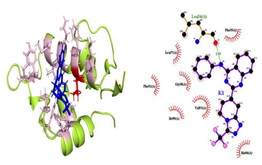


Confocal microscopy images of AGS and MCF7 cells treated with K1 20μM for 72hr showing expression of p53 and MDM2. (a) In AGS cells, p53 expression(green) is increased in the cytoplasm in presence of K1 and MDM2(red) expression is downregulated with K1 treatment. (b) In MCF7 cells, p53 expression is increased in the nucleus and MDM2 expression is downregulated with K1 treatment. Merged images shows the colocalization of p53 and MDM2. Bar graph showing the varied expression of p53 and MDM2 in AGS (c) and MCF7 (d) cells

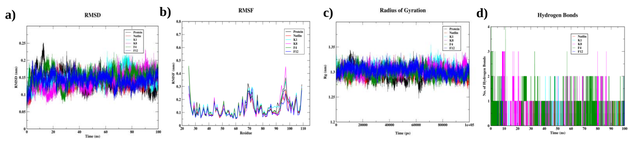
Molecular docking of K1 and MDM2

Table 1. Comparison of docking score (Glide and GOLD), X-Score and LigPlot interactions of nutlin and selected small molecules

Compounds	No. of Hydrogen bond interactions	Interacting residues	No. of Non Bonded Interactions	No. of Hydrophobic interactions	Glide score (kcal/mol)	Glide X-score (kcal/mol)	Gold Fitness Score	Gold Xscore(kcal/mol)
Nutlin	2221	His 96	29	8	-7.04	-9.3	51.86	-9.13
K1	1	Leu 54	29	7	-6.9	-8.41	50.12	-8.42
K8	1	Leu 54	27	7	-5.5	-8.1	47.44	-8.32
F4	2	Gln 72,Leu 54	22	6	-6.5	-8	52.07	-8.03
F12	0	0	27	9	-5.04	-7.62	51.05	-8.09

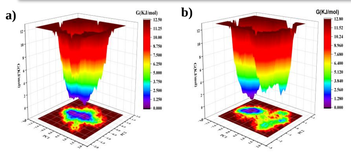


Molecular Dynamics Simulation



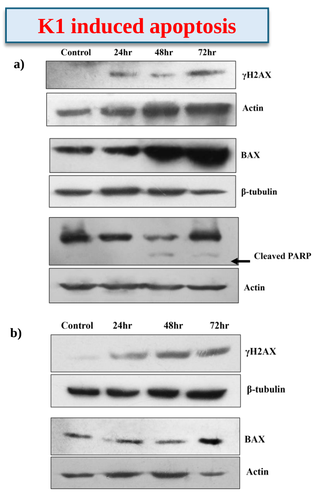
MD simulation data analysis plots: (a) root mean square deviation (RMSD) plot of backbone atoms (b) root mean square fluctuation (RMSF) plot of Cα atoms of protein residues (c) Radiation of Gyration plot over time and (d) No. of H-bonds averaged over time of approved and selected compounds. Protein (Black), nutlin (Red), K1 (Cyan), K8 (Violet), F4 (Green) and F12 (Blue).

Free Energy Landscape Plot



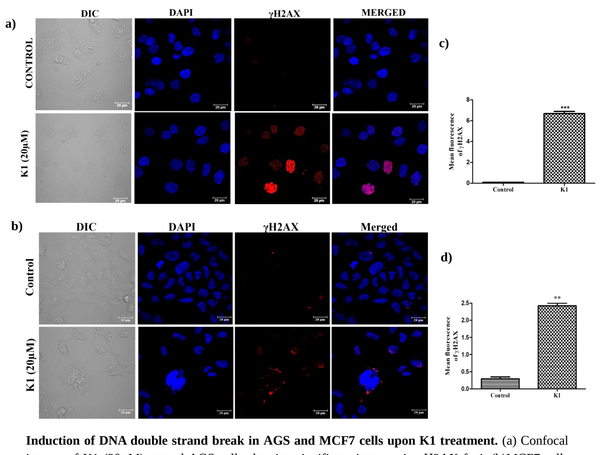
Gibbs free energy landscape plots of (a) **MDM2-Nutlin** (b) **MDM2-K1**. The free energy is given in kJ/mol and indicated by colour code panel from lower to higher.

K1 induced apoptosis



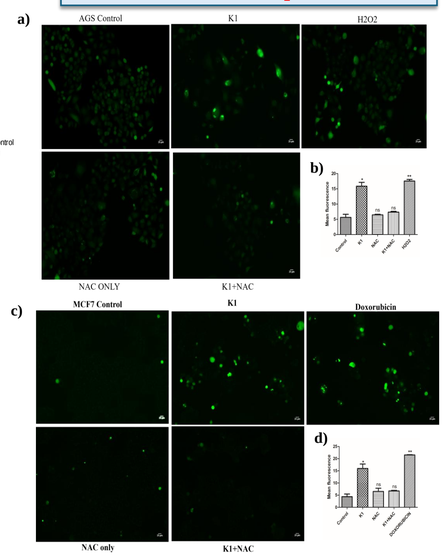
Induction of DNA damage and apoptosis in AGS and MCF7 cells upon K1 treatment. (a) In AGS cells upregulation of γH2AX, BAX and cleavage of PARP protein levels with K1. (b) In MCF7 cells treated with K1 increased level of γH2AX and BAX protein expression.

K1 induced DNA damage in AGS cells



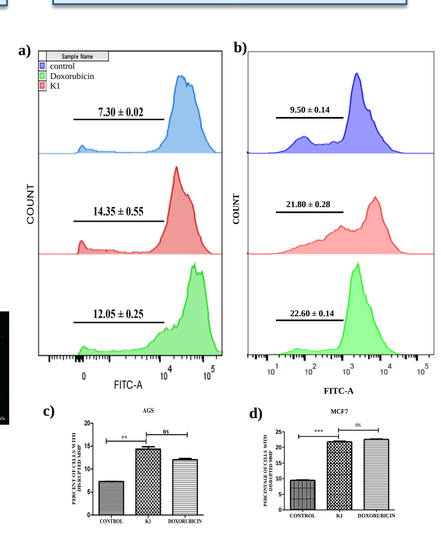
Induction of DNA double strand break in AGS and MCF7 cells upon K1 treatment. (a) Confocal images of K1 (20μM) treated AGS cells showing significant increase in γH2AX foci. (b)MCF7 cells treated with K1 (20μM) shows increased level of γH2AX expression. (c) and (d) Graph showing significant increase in the mean fluorescence with K1 treatment for 72hr in AGS and MCF7 respectively.

K1 activated ROS production



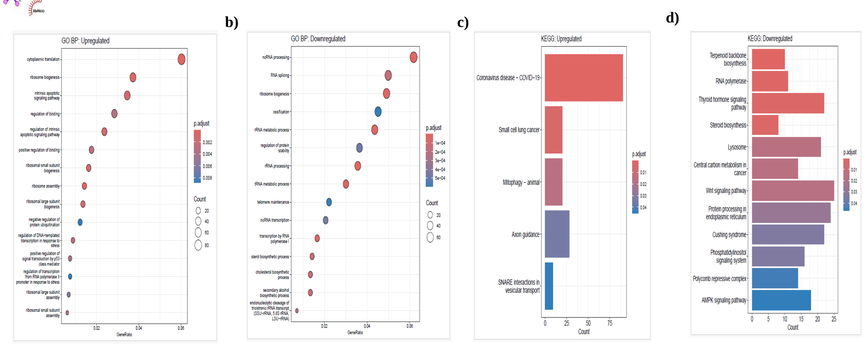
K1 increases ROS production in AGS and MCF7 cells. (a) Fluorescent microscopy images of AGS cells treated with K1, H_2O_2 NAC or both NAC and K1. (b) Bar graph indicates an increased mean DCF fluorescence intensity in K1 and H_2O_2 treated AGS cells. (c) Fluorescent microscopy images of MCF7 cells treated with K1, doxorubicin NAC or both NAC and K1. (d) Bar graph indicates an increased mean DCF fluorescence intensity in K1 and doxorubicin treated MCF7 cells. **P< 0.01 and *P< 0.05 as compared to DMSO treated control.

K1 induced dissipation of MMP

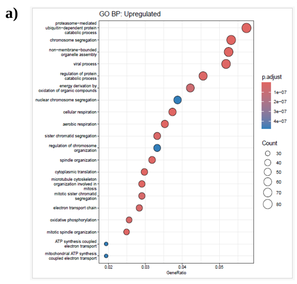


K1 increases dissipation of MMP in AGS and MCF7 cells. Decreased fluorescence uptake in AGS (a) and MCF7 (b) cells treated with K1 as compared to control. Bar graph indicates increased present of dissipation of MMP in AGS (c) and MCF7 (d) cells post K1 treatment. **P< 0.01 and *P< 0.05 as compared to DMSO treated control.

Transcriptomic Profiling and Functional Annotation

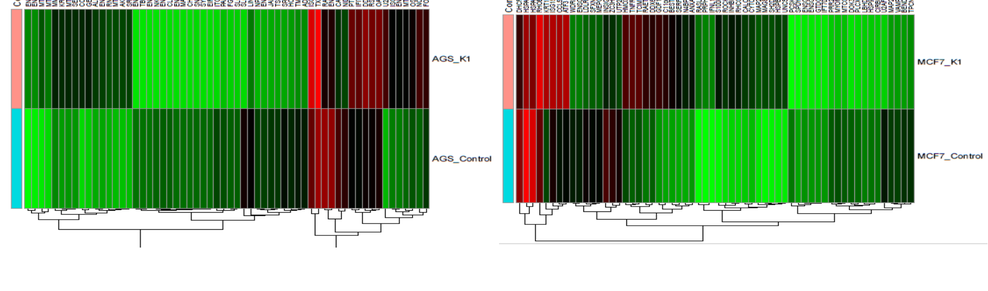


Gene Ontology and KEGG enrichment. GO plot for differentially expressed genes in MCF7 cells treated with K1 as compared to control. (a) GO plot for upregulated genes and (b) for downregulated genes. KEGG pathway for the upregulated genes (c) and downregulated genes (d). Statistical significance of differential expression was determined with edgeR, from adjusted $p < 0.05$ considered significant.



Venn diagram showing overlap of differentially expressed genes. Venn diagram illustrating the number of differentially expressed genes between control and K1 treated groups of AGS(pink color) and MCF7(green color) samples.

Gene Ontology and KEGG enrichment. GO plot for differentially expressed genes in AGS cells treated with K1 as compared to control. (a) GO plot for upregulated genes and (b) for downregulated genes. KEGG pathway for the upregulated genes (c) and downregulated genes (d). Statistical significance of differential expression was determined with edgeR, from adjusted $p < 0.05$ considered significant.



Heatmap of differentially expressed genes from RNA sequencing analysis. Heatmap displaying differentially expressed upregulated and downregulated genes between control and K1 treated AGS (a) and MCF7 (b) cells. Each row represents a gene, and each column represents the samples. Color intensity corresponds to relative expression levels, with red indicating upregulation and green indicating downregulation.

CONCLUSION AND FUTURE DIRECTION

K1 inhibits cell proliferation, clonogenicity and changes the morphology of gastric cancer cell line AGS and breast cancer cell line MCF7. K1 decreases p53 and MDM2 expression in AGS while in MCF7 cells, p53 expression is upregulated and MDM2 get downregulated. K1 induced cell cycle arrest at G2/M for AGS and G1 for MCF7 cells. ROS production and MMP dissipation by K1 in both cell lines initiate the DNA damage, increased expression of γH2AX, cleaved PARP, BAX leading to apoptosis. Validation genes from transcriptomic analysis will help to know pathways and genes involved in the differential expression of K1 in AGS and MCF7 cells.

ACKNOWLEDGEMENT

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