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Summary and Conclusions
6.a. Summary

For the first time, Mdm2 and Pirh2 promiscuous binding small molecule modulators have been identified by extensive in silico screenings and in vitro evaluations. First, the crucial Mdm2 and Pirh2 interacting residues of p53TAD and p53TET, respectively have been delineated from the literature, sequence conservation analysis as well as from the protein-protein docking and molecular dynamics simulations. The 3D structural information of crucial residues have been used as the benchmark to construct Mdm2 and Pirh2 focused ligand libraries; created by the pharmacophore-based screening of 3.9 million compounds deposited in MMsINC® database. Extensive in silico screening exercises have resulted in twelve potential best-fit molecules which displayed favorable binding interactions with the both Mdm2 pBD and Pirh2 CTD.

90 structural analogues pertaining to MMs02943764, MMs03738126 and MMs02995593 (best-fit molecules) were generated and docked to both Mdm2pBD and Pirh2CTD. Of the 90 analogues, thirteen analogues were subjected to MD simulations using Mdm2pBD and Pirh2CTD as target proteins in explicit waters. 27 molecules including MMs02995593 were synthesised and characterised in our laboratory. Of the 27 analogues, PAC and PAA displayed greater binding affinity towards Mdm2 and Pirh2 during the in silico evaluations. Both the molecules also exhibited good antiproliferation activity in vitro against MCF7 (IC\textsubscript{50} = 61.58 µM (PAC), 61.59 µM (PAA)), Reh (IC\textsubscript{50} = 5.27 µM (PAC), 4.26 µM (PAA)), Nalm6 (IC\textsubscript{50} = 19.61 µM (PAC), 35.86 µM (PAA)), and K562 cancer cell lines (IC\textsubscript{50} = 35.264 µM (PAC), 54.404 µM (PAA)). Further PAC induced growth arrest at SubG0/G1, S and G2 phases in K562 cells. Flow cytometric analysis revealed PAC suppresses the expressions of Mdm2 and Pirh2 proteins which caused higher expression level of p53 in K562 p53 null cells.

MMs02995593, which is among the 12 best-fit molecules displayed poor binding towards the Mdm2 and Pirh2 during MD simulations. It also exhibited poor cytotoxicity on the tested cancer cell lines. Structural comparisons revealed that intramolecular π-π stacking interaction between imine functionality and the phenyl ring makes MMs02995593 a poor Mdm2 and Pirh2 binding candidate. The structural optimisation of MMs02995593 has resulted in TTP, which would be a promising and high affinity Mdm2 binding new hit molecule. Future studies are warranted for further structural optimisation of PAC, PAA and TTP to improve in vitro and in vivo efficacy.

Overall, these findings would inspire and attract researchers and oncologists from different parts of the world for the development of highly potent Mdm2 and Pirh2 promiscuous inhibitors which has implications in cancers prone to develop chemo-resistance.
6.b. Conclusions

Computational tools have been successfully employed to delineate the p53TET binding orientation on the Pirh2CTD. The p53TET_Or2 residues viz. L330, M340, F341 and L344 are found to interact with Pirh2CTD. Whereas, p53TAD residues such as, F19, W23 and L26 are crucial for Mdm2pBD binding. Computational analyses were also employed for the first time to identify potential promiscuous modulators of Mdm2 and Pirh2 E3(Ub)-ligases. Twenty-seven in silico designed analogues were synthesised in the laboratory and extended to the in vitro biological testing. Two molecules (PAC, PAA, which are structural analogues of primary shortlisted hit candidates MMs02943764, MMs03738126) displayed good anti-proliferation activity against four different cancer cell lines (MCF-7, Reh, Nalm6 and K562). Furthermore, PAC displayed cytotoxic potency by inducing the growth arrest at the sub G0/G1 and S phases of the cell cycle in K562 cells. Flow cytometric analysis revealed that PAC induces the p53 expression level and subsequently decreases the expression levels of Mdm2 and Pirh2 proteins in K562 cells. On the other hand, TTP which is originated from the structural optimisation of MMs02995593 displayed similar Mdm2 binding property as the three-point pharmacophore residues of p53TAD (Phe19, Tyr22, and Trp23). More studies are warranted to carry out structural optimisation of the shortlisted hit candidates in the realm of bioactivity, which would be later extended to preclinical and clinical evaluations in the near future.