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Interaction studies to evaluate 2- carboxyphenolate analogues as inhibitor of anti-apoptotic protein Bcl-2

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Abstract:
Apoptosis is a cellular process that leads to the death of damaged cells. Its malfunction can cause cancer and poor response to conventional chemotherapy. After being activated by cellular stress signals, pro-apoptotic proteins bind anti-apoptotic proteins, thus allowing apoptosis to go forward. An excess of anti-apoptotic proteins can prevent apoptosis. Designed molecules that imitate the roles of pro-apoptotic proteins can promote the death of cancer cells. In this work we have applied an insilico approach to study the binding of 2-carboxyphenolate analogues as potent inhibitors of anti-apoptotic protein Bcl-2. Molecular docking study was performed in order to find specific binding mode using AutoDock. From the docking results it was observed that zinc 2-carboxyphenolate showed strong inhibition with Bcl-2 with docking energy of -4.6 kcal/mol. The effects of the Zinc 2-hydroxybenzoate on apoptosis in HT-1080 cell lines were also analysed, which shows strong evidence for their apoptotic mode of action using flow cytometric analysis of Annexin-V. Our study gave valuable insights on inhibitor specificity of anti-apoptotic proteins and might be considered as potent chemopreventive agents.

Keywords: Apoptosis, Docking, Bcl-2, AutoDock, 2-carboxyphenolate analogues, 2-hydroxybenzoate analogues.

Background:
Cancer is one of the most leading causes of death worldwide, accounting for 7.6 million deaths in 2008 **[1].** Major cause for cancer is restriction of programmed cell death that is called apoptosis. Apoptosis is arguably the most potent natural defence system against cancer, because it eliminates premalignant cells that enter in S phase inappropriately after genetic sabotage of restriction point controls **[2].** It is one of the major mechanisms of cell death in response to cancer therapies **[3].** Alterations in susceptibility to apoptosis can enhance resistance to conventional anticancer therapies, such as radiation and cytotoxic agents [4]. One of the mechanisms of resistance to cytotoxic agents is the alteration in expression of B-cell lymphoma-2 (Bcl-2) family members, which are responsible for maintaining a balance between newly forming cells and old dying cells. The Bcl-2 family contains both anti- and pro-apoptotic proteins with opposing biological functions in either inhibiting or promoting cell death **[5].** These family members can be divided into three subfamilies based on structural and functional features. The anti-apoptotic subfamily includes the Bcl-2, Bcl-XL, Bcl-w, Mcl-1, Bfl1/A-1, and Bcl-B proteins, which suppress apoptosis **[6].** When anti-apoptotic Bcl-2 family members are overexpressed, the ratio of pro- and anti-apoptotic Bcl-2 family members is disturbing and can causing prevention in apoptotic cell death. Inhibiting the anti-apoptotic Bcl-2 family of proteins can improve apoptosis and thus overcome drug resistance problem to cancer chemotherapy **[7, 8, 9].**

For a successful cancer therapy, we require drugs that will activate apoptosis in tumours, with minimal side effects on normal cells. To do these, we have computationally design and

develop analogues of 2- carboxyphenolate and dock with anti-apoptotic Bcl-2 protein. Interaction between the Bcl-2 protein with 2- carboxyphenolate derivatives were analysed and predicted that zinc 2- carboxyphenolate has greater binding affinity with Bcl-2 protein. Further, we experimentally confirm the apoptotic effects of zinc 2- carboxyphenolate on HT-1080 Cell Lines.

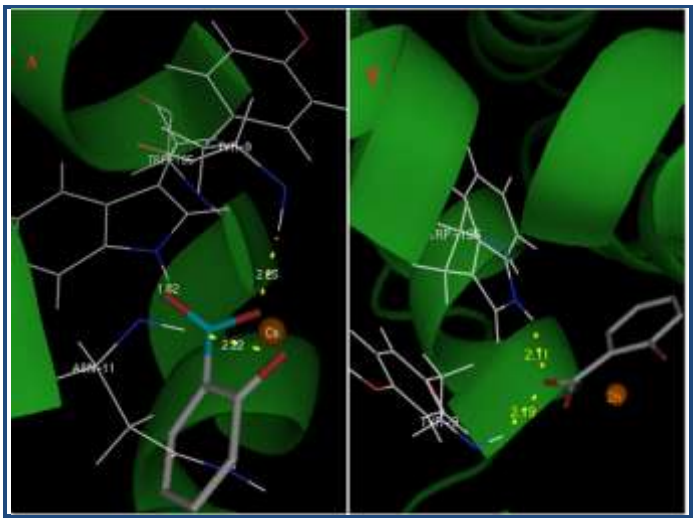

Figure 1: (a) The docking pose of complex between Bcl-2 protein and Calcium 2- carboxyphenolate; **(b)** The docking pose of complex between Bcl-2 protein and Zinc 2-carboxyphenolate. Hydrogen bond between inhibitor and residue is represented by dotted yellow line in each case.

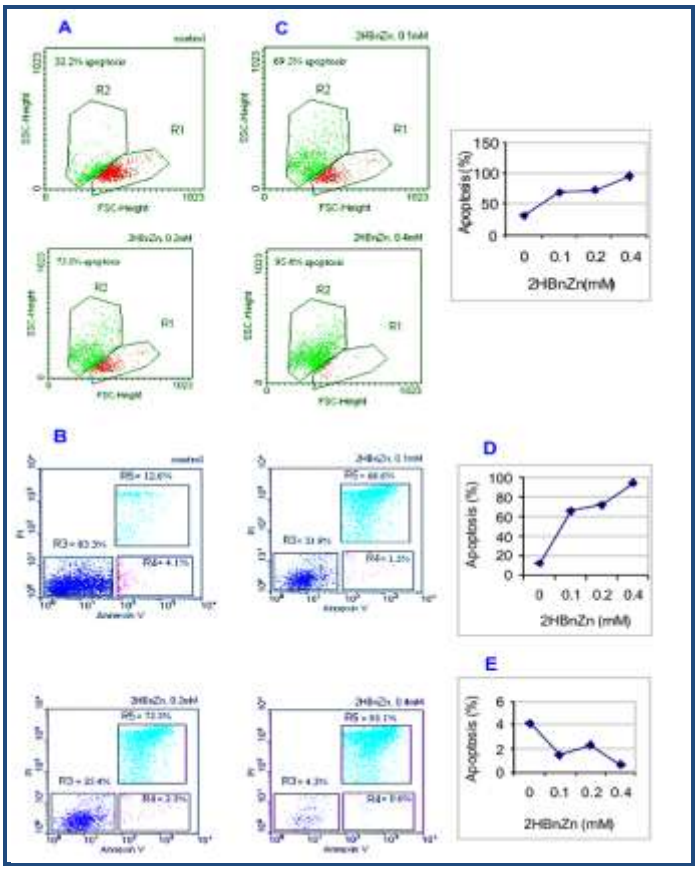

Figure 2: FACS results of the concentration-dependent increase in **(A)** propidium iodide (PI) and **(B)** Annexin-V (An-V) labelling of HT-1080 cells treated with 0.1, 0.2 and 0.4mM zinc 2-hydroxybenzoate or Zinc 2-carboxyphenolate for 24 hours. **(C)** % of early and late apoptotic cells labelled by PI (Green), **(D)** % of late apoptotic cells labelled by An-V (blue), and **(E)** % of early apoptotic cells labelled by An-V (red). Cells were cultured under standard conditions, arrested with serum free DMEM medium, treated with zinc benzoate at the indicated concentration points and labelled by FITC-labelled Annexin V.

Methodology:

The 3D coordinates of the solution structure of the anti-apoptotic protein Bcl-2 (PDB ID: 1G5M) in Homo sapiens was retrieved from Protein Databank (http://www.rcsb.org/). The 2D structures of 2-carboxyphenolate or 2-hydroxybenzoate analogues were constructed using ChemSketch [**10**] and converted in to 3D structure using OpenBabel **[11]** tool. All the ligands were subjected to energy minimization and molecular dynamics using the HyperChem software **[12].** Energy calculations were carried out using the AMBER force field. Molecular structure optimization of ligands were carried out using conjugate gradient method Polak-Ribiere until the maximum energy derivative was lower than 0.1kcal/Å mol in order to obtain a correct geometry.

Molecular Docking

Docking of all 2-carboxyphenolate analogues with Bcl-2 protein structure was carried out using AutoDock v3.0.5 [**13, 14**]. Gasteiger charges were added to the ligand and maximum 6 number of active torsion are given to the lead compound using AutoDock Tool **[15]**. Kollaman charges and solvation term were added to the protein structure using AutoDock Tool. The Grid for docking calculation was centered to cover the protein binding site residues and accommodate ligand to move freely. During the docking procedure a Lamarckian Genetic Algorithm (LGA) were used for flexible ligand rigid protein docking calculation. Docking parameters were as follows: 30 docking trials, population size of 150, maximum number of energy evaluation ranges of 25,0000, maximum number of generations is 27,000, mutation rate of 0.02, cross-over rate of 0.8, Other docking parameters were set to the software's default values.

Cells and in vitro culture Conditions

HT-1080 cells were cultured in an optimal DMEM medium and under a humidified atmosphere of 95% air and 5% CO_2 at 37°C using standard suitable culture vessels. HT-1080 cells were seeded at specific density unless otherwise and allowed to grow for 3 days followed by 24 hours culturing in serum-free DMEM medium before being treated with 2-hydroxybenzoate analogues individually at specific concentration(s) and for certain time(s). Cells were harvested and centrifuged at 1000g for 5 minutes before conducting the test by specific technique.

Flow Cytometric analysis of Apoptosis

HT-1080 cells were seeded in T-25 flask at an initial density of $1x10^3$ per flask and cultured for 3 days before the treated with zinc 2-hydroxybenzate (0.1, 0.2 and 0.4mM) for 48 hours. At the end of the treatment, $1x10^6$ cells were harvested by centrifuged and then washed with phosphate buffered saline. Cells were resuspended in 100µl of binding buffer and 1µl (final concentration 1mg/ml) of FITC-labelled Annexin-V (Dako cytomation, UK) and then incubated in the dark for 10 minutes at room temperature. 100µl (final concentration 1mg/ml) of

propidium iodide solution (Dako cytomation, UK) was added to each aliquot prior to analysis by flow cytometry using CellQuest software on a FACSCalibur flow cytometer (Becton Dickinson, UK).

Results and Discussion:

Docking studies predicted the interaction of ligand with protein and residues involved in this complex. For such interaction studies, the most important requirement was the proper orientation and conformation of ligand which fitted to the enzyme binding site appropriately and formed protein-ligand complex. Therefore, optimal interactions and the best AutoDock score were used as criteria to interpret the best conformation among the 30 conformations, generated by AutoDock program. All the 2-carboxyphenolate analogues were docked in to structure of Bcl-2 protein. The docking results of 2-carboxyphenolate and its analogues were shown in **Table 1 (see supplementary material).** Among the above docked compounds Zinc 2-carboxyphenolate had the lowest docking energy (-4.6 kcal/mol) with Bcl-2 protein. Docking poses of the best conformation of Calcium 2- carboxyphenolate and Zinc 2- carboxyphenolate in binding site of Bcl-2 protein were shown in **(Figure 1 a, b).** Residues of protein involved in the formation of hydrogen bonds with these compounds are TYR9, ASN11 and TRP195.

Detection of Apoptosis via Annexin-V

In order to quantify the effect of benzoate analogues on the induction of apoptosis in HT-1080 cells, double staining for both FITC-labelled Annexin-V binding and cellular DNA using propidium iodide were used and measured by the flow cytometry to substantiate the morphological results that showed zinc benzoate analogue mostly induced apoptosis compared to others. Annexin-V was used to measure surface changes through the labelling of the externalised phosphatidylserine (PS) when the cell is committed to the apoptosis pathway of death. **Figure 2a** shows the percentage of late apoptotic (or the secondary necrotic) HT-1080 cells cultured in DMEM optimal growth medium in the presence of zinc 2-hydroxybenzoate for 24 hours. These results clearly indicate that the zinc analogue induced apoptosis in a concentration-dependent manner, as labelled by propidium iodide (PI) (the red fluorescence). The percentages of positively stained cells with PI increased 2.2 fold, 2.5 fold and 3 folds when the HT-1080 cells were exposed to 0.1, 0.2 and 0.4mM zinc 2-hyroxybenzoate respectively. Furthermore, the FITC-labelled Annexin-V and propidium iodide double staining show the viable cells (negative for both AV and PI, shown in dark blue dots), the early apoptotic cells (stained + AV and – PI, shown as red dots) and the secondary necrotic, or late apoptotic cells (stained positive for both AV and PI, shown as light blue dots) cells and gives both early and late apoptotic HT-1080 cells **(Figure 2b)**. As with the PI positively stained cells, the late apoptotic cells also increased by 5.3 fold, 5.7 fold and 7.5 fold when HT-1080 cells exposed respectively to 0.1, 0.2, and 0.4mM of zinc analogue. These results are in agreement with the corresponding morphological experiment with annexin-V. Other morphological techniques showed no HT-1080 necrotic cells.

Conclusion:

Inhibition of the interaction between Bcl-2 (anti-apoptotic protein) and pro-apoptotic proteins could be an important step that leads to apoptosis. Therefore, the discovery of compounds with the capacity to inhibit Bcl-2 is an ongoing research topic on cancer therapy. The approach utilized in this study is successful in finding Zinc 2- carboxyphenolate as potent Bcl-2 inhibitor. Docking study predicted that Zinc 2-carboxyphenolate has lowest docked energy (-4.6 kcal/mol) with Bcl-2 protein and interaction is stabilizes by hydrogen bonding. Hydrogen bonding plays an important role for the structure and function of biological molecules, especially for inhibition in a complex. Further, the work was evaluated experimentally to study the effects of the Zinc 2-carboxyphenolate on apoptosis in HT-1080 cell lines. The flow cytometric analysis of Annexin-V showed evidence of apoptosis. The evidence of apoptosis may indicate that the induction of cell death is by a mechanism that does not involve a specific phase arrest.

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Supplementary material:

Table 1: The docking results of 2-carboxyphenolate and its analogues with anti-apoptotic Bcl-2 protein.

Sl. No	CID No.	IUPAC Name	Binding Energy (Kcal/mol)	Docking Energy (Kcal/mol)	Intermol Energy (Kcal/mol)	Torsional Energy (Kcal/mol)	Internal Energy (Kcal/mol)
1	54675850	2-carboxyphenolate	-2.77	-3.2	-3.08	0.31	-0.11
2	54690287	Calcium 2-carboxyphenolate	-2.78	-3.56	-3.4	0.62	-0.16
3	54746096	Zinc 2-carboxyphenolate	-4.6	-4.6	-4.6	0.0	0.0
4	54683737	Silver 2-carboxyphenolate	-2.8	-3.5	-3.42	0.62	-0.08
5	23698465	Sodium 2-carboxyphenolate	-2.12	-2.83	-2.74	0.62	-0.09
6	54725519	2-carboxyphenolate; manganese(2+)	-4.18	-4.18	-4.18	0.0	0.0
7	54723200	Magnesium 2-carboxyphenolate	-4.72	-4.72	-4.72	0.0	0.0
8	54713150	Copper 2-carboxyphenolate	-3.45	-4.2	-4.07	0.62	-0.13