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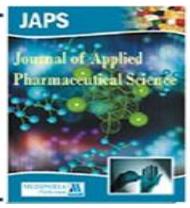

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Withaferin A targets apoptosis inhibitor cIAP1: A potential anticancer candidate

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ABSTRACT

Inhibitors of Apoptosis (IAPs) are important and well studied molecules in the cancer pathway. These are known inhibitors of apoptosis and are over expressed in many tumours where they confer chemo-resistance. The cIAP1 inhibits caspase 3 and caspase 7 and ensures suppression of apoptosis. Docking studies carried out with herbal ligand withaferin A derived from roots of *Withania somnifera* onto the structure of cIAP1. Withaferin A has shown strong binding affinity of -15.4988 kJ/mol with BIR domain of cIAP1 and in turn interfere in the binding of cIAP1 molecule to caspase 3 and caspase 7 and thus result into prevention of inhibition of these effecter proteins. The docking studies support the experimental outcomes and suggest that withaferin A has potential to develop as candidate molecule for cancer therapy.

Keywords: Apoptosis, Inhibitors of apoptosis, *Withania somnifera*.

INTRODUCTION

There is an indispensable need for exploration of novel natural products as anti-cancer drug candidates. Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer, in addition to other diseases including neurodegeneration, coronary human diseases including diabetes (Hunter *et al.*, 2007). In a healthy organism, cells typically die via apoptosis; however, the uncontrolled cell proliferation characteristics of most cancer cells require that apoptosis be suppressed. As solid tumor continues to grow, the resulting microenvironment often becomes deficient of growth factors, and lacks adequate oxygen supply. Although these conditions would trigger apoptosis in normal cells, cancer cells continue to proliferate (Hanahan and Weinberg 2000, Evan and Vousden 2001). Inhibitors of apoptosis proteins (IAPs), also known as a baculovirus IAP repeat (BIR) - containing proteins (BIRCs), are evolutionarily conserved proteins defined by structural similarity. They share one to three copies of a well conserved domain of about 70 amino acids named BIR (Dubrez-Daloz *et al.*, 2008). The IAPs effectively suppress apoptosis induced by a variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, and genotoxic damage (Hunter *et al.*, 2007).

There is direct evidence that IAPs are able to function as oncogenes. This is seen in the case of cIAPs where chromosome amplification of the 11q21-q23 region, encompassing cIAP1, has been observed in variety of malignancies, including medulloblastomas, renal cell carcinomas, glioblastomas, gastric carcinomas and non-small cell lung carcinomas. Esophageal squamous-cell carcinomas frequently display this amplification, and transcriptional profiling has identified cIAP1 as sole target that is consistently over-expressed in these tumors (Imoto *et al.*, 2001). Inhibitor of apoptosis proteins (IAPs) regulate apoptosis primarily by inhibiting caspase-family proteases. However, many IAPs also possess E3 ligase (ubiquitin-protein isopeptide ligase) activities implicated in both caspase-dependent and -independent functions of these proteins. The N-terminal (BIR1) and C-terminal (BIR3) BIR domains of cIAP1 are necessary and sufficient for binding TRAF2 and SMAC, respectively. Mutational analysis of the BIR1 and BIR3 domains identified critical residues required for TRAF2 and SMAC binding. Several mammalian IAP-family proteins have been reported to bind SMAC, including XIAP, cIAP1, cIAP2, and ML-IAP. The structural basis for the interaction of SMAC with IAP-family proteins has been determined for XIAP, where NMR, x-ray crystallographic, and other supporting studies have revealed a tetrapeptide motif in the N terminus of proteolytically processed SMAC that binds a surface crevice on the BIR3 domain of XIAP. Homology modeling and sequence analysis suggest that the BIR3 domain of cIAP1 is very similar to XIAP, with conservation of 8 of the 11 residues in XIAP that have been determined to account for interactions with the N terminus of mature SMAC. (Samuel *et al.*, 2006). Beside these IAPs also modulate apoptosis by binding and inactivating caspases. Inhibitor of apoptosis proteins (IAPs) interact with and inhibit caspases-3, -7, and -9. This interaction can be inhibited by Smac/DIABLO, a polypeptide released from mitochondria upon initiation of the apoptotic signaling process (Arnt *et al.*, 2002).

The realization that alterations in inhibitor of apoptosis (IAP) proteins are found in many types of human cancer and are associated with chemoresistance, disease progression and poor prognosis, has sparked a worldwide frenzy in the development of small pharmacological inhibitors of IAPs (Gyrd-Hansen & Meier, 2010).

In earlier reports, *Withania somnifera* root extracts and several withanolides isolated from it showed the inhibition of human colon, lung, CNS and breast cancer cell proliferation (Anjaneyulu *et al.*,, 1997, Jayaprakasam *et al.*, 2003, Grover *et al.*, 2010, 2011). We have earlier reported the production of withanolides in root organ culture of *Withania somnifera* under define environmental conditions (Wadegaonkar *et al.*, 2006).

A detailed understanding of how cIAP1 interacts with chemical inhibitors that modulate their activity in therapeutically useful ways. We, therefore, undertook computational approach to predict and validate the effect of withaferin A (an active anticancer component of in vitro grown roots of *Withania somnifera*) on cIAP1. These studies demonstrate that inhibition of IAP proteins can modulate the efficacy of antineoplastic agents.

METHODS AND MATERIAL

Ligand and Receptors

The crystal structure of cIAP1-BIR3 domain in complex with the Smac-mimetic compound Smac037 [PDB: 3MUP] (Cossu *et al.*, 2010) was obtained from the Protein Data Bank (PDB) (Berman *et al.*, 2000). The ligand molecule withaferin A [PubChem: 265237] was retrieved from NCBI PubChem Compound database. The structure of this compound is shown in Figure 1. The energy of the ligand molecule was minimized by AMBER force field using VLife Engine and saved as Mol2 file format.

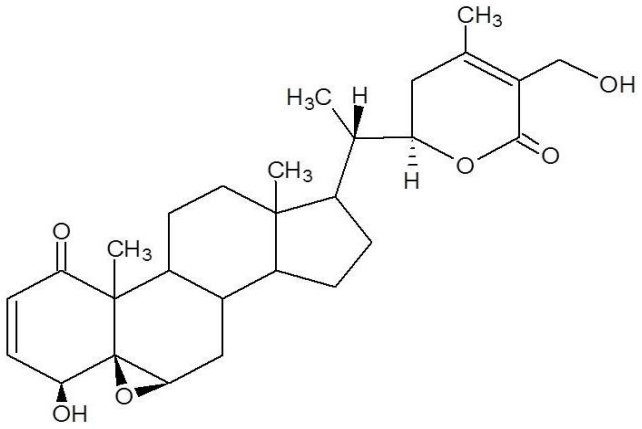

Fig. 1: Chemical structure of Withaferin A.

Structural aspects of cIAP1-BIR3 domain in complex with the Smac-mimetic compound Smac037

The structural features of the receptor macromolecule considered in our study [PDB: 3MUP] have been described in detail elsewhere (Cossu *et al.*, 2010) by the depositors of the crystal structure to the Protein Data Bank. Briefly, the structure includes the BIR3 domain of cIAP1 in complex with Smac037. Thermal stability and fluorescence polarization assays show the stabilizing effect and the high affinity of both Smac037 and Smac066 for cIAP1-BIR3 domains. The BIR3 domain interacts with Smac037 (residues 254-355) comprises of 8 helices (49 residues) and 3 strands (beta sheets – 8 residues).

Docking

Drug and ligand binding was simulated as protein ligand interaction using software LeadIT (BioSolve IT, GmbH). FlexX is an analysis scheme that was used which breaks the ligand into fragments, then repeatedly place an anchor fragment and incrementally build the entire ligand in place. FlexX uses a variant of the SCORE1 scoring function developed by Hans-Joachim Boehm for the de novo enzyme inhibitor design package LUDI (Boehm, 1992).

Since the 3-D structure of ligand-protein complex was available, it was taken as reference ligand and binding site was defined such a way that it will include all amino acid residues within 6.5˚A radius. The values of protonation of polar groups in binding site of cIAP1 are shown in Table 1.

The triangle matching method was used for docking of Withaferin A as ligand to the cIAP1 protein to defined binding site and analyze on the basis of free binding energy, hydrophobic interaction and hydrogen bonds between ligand and receptor molecules.

Table. 1: Protonation of polar groups in the binding site of 2MUP (cIAP1).

Amino acid	Protonation state	Torsion
Arg-294A	arg+	-
Asp-296A	asp-	--
Asp-297A	asp-	--
Lys-299A	lys+	180
Asp-304A	asp-	--
Arg-308A	arg+	--
Cys-309A	cys-	--
Glu-311A	glu-	--
Asp-314A	asp-	--
Glu-319A	glu-	--
Lys-322A	lys+	180

RESULT AND DISCUSSION

Biochemical analysis has clearly established the fact that during apoptosis, Smac is released into the cytosol together with several other mitochondrial proteins (apoptosis inducing factors). The mature form of Smac binds to several IAPs and removes the ability of IAPs to block caspase-mediated apoptosis (Wu *et al.*, 2000). Withaferin A inhibits the proliferation of various cancer cells, therefore it was of interest to see whether withaferin A molecule interact with cIAP1 molecule for binding site of Smac and its mimetic compounds. LeadIT ver 1.3.0 was used to predict interaction of Withaferin A with cIAP1. LeadIT uses FlexX technology and has user friendly graphical interface to prepare, launch and analyse dock run. For understanding the mechanism of inhibition by Withaferin A, cIAP x-ray crystallographic structure complex with inhibitor (PDB ID 3MUP) available at RCSB protein data bank was used. The structure 3MUP has four identical polypeptide chains with 122 amino acid residues. This protein was co-crystallized with Smac037, a Smac-mimetic compound (Cossu *et al.*, 2010). This inhibitor was used a\s reference ligand for docking studies. The reference ligand has hydrophobic interaction with Trp323, Leu354, Leu307 and Gly306 and hydrogen bonds with Arg208 and Asp314 of BIR region (Fig 2). The binding site was defined by including amino acid residues present with in 6.5 A radius around this interaction site of reference ligand.

One possible mode of action which is proposed here for Withaferin A to modulate activity of cIAP1 by occupying the Smac specific binding pocket present in the BIR region of the cIAP1 molecule. As FlexX reports the best docking solution for each run and also performs a cluster analysis in which the total number of clusters and the rank of each docking mode (cluster rank) is reported, in 9 out of 10 docked conformations obtained by the clustering analysis at 6.5 Å, the epoxy group of the lactone ring is found closest to the amino group of Lys322 (Figure 3). The binding energies of the conformations of this cluster range from -1.8559 to -15.4988 Kcal/mol.

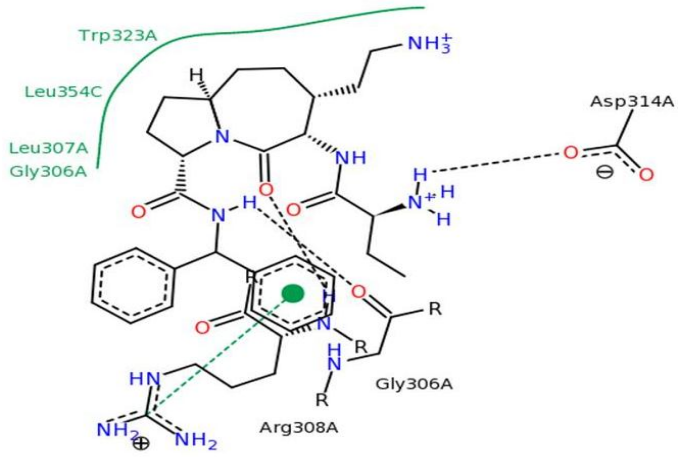

Fig. 2a: Interaction of Smac037, a Smac-mimetic compound with cIAP (PDB ID 3MUP).

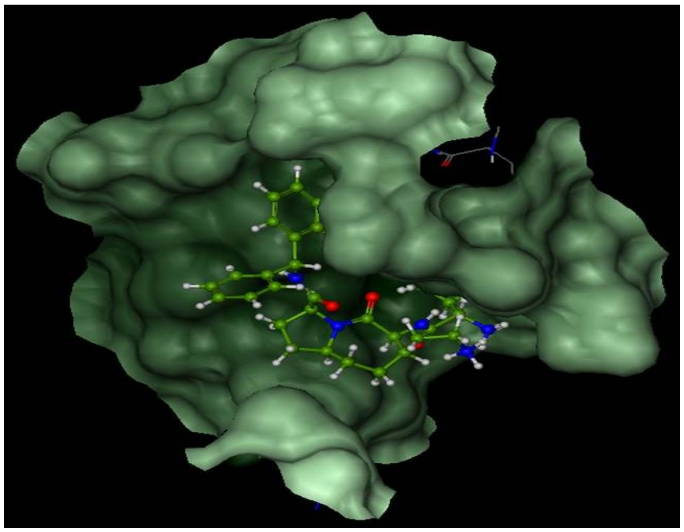

Fig. 2b: Docking of Smac037 Legand: black dashed lines - hydrogen bonds; green solid lines - hydrophobic interactions.

FlexX when used to analyse the interaction between cIAP1, Smac mimetic compound and inhibitor (Withaferin A, component of methanolic extract of *Withania somnifera*), The binding of Withaferin A to cIAP1 is characterized by H-bondings between a terminal hydroxyl group of the ligand and the side chain carboxyl group of Asp314, and epoxy group of ligand and –NH group of indole ring of Trp323 and ε-amino group of Lys322 (Figure 3 A.). The lengths of the H-bonds formed are 2.66, 1.92 and 1.92 Å respectively. It has also been reported that the residues Leu307, Arg308, Cys309, Asp314, Trp323 and Glu319, of cIAP1 are responsible for intermolecular hydrophobic interactions. Withaferin A showed strong binding affinity of -15.4988 kJ/mol with cIAP1 in the active site. Withaferin A docked into the same cavity in which Smac mimetic compound docked with hydrophobic interactions with Cys 309, Trp323, Glu319. in the BIR region of cIAP1(Fig. 3).

Our docking results obtained substantiate the hypothesis that Withaferin A has the potential to inhibit the association of cIAP1 to Caspase3 and Caspase7 by binding to BIR region of cIAP1. Since Withaferin A is a small herbal molecule, it is expected to provide one of the modest modes of treatment along with added favours of ease in oral administration and decreased immunogenicity. Conclusively it is strongly suggested here that Withaferin A is a potent molecule which can disturb the binding of cIAP1 with caspases and can activate apoptosis in cancer cell. Withaferin A should be looked forward for further clinical investigations as a possible IAP inhibitory drug candidate.

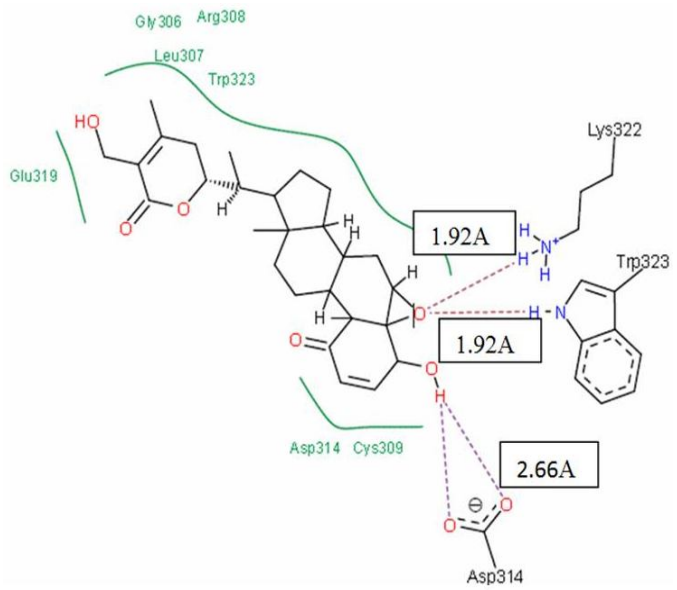

Fig. 3a: Interaction of Withaferin A with cIAP1 (PDB ID 3MUP).

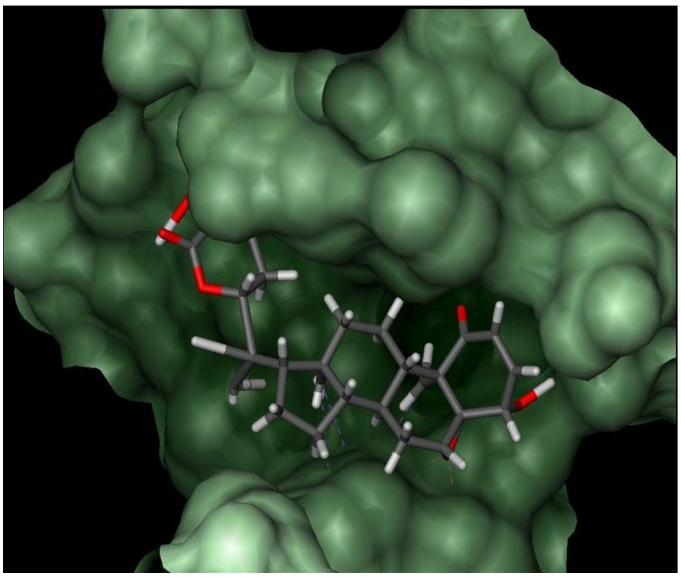

Fig. 3B: Docking of Withaferin A into the cavity of cIAP1. Reference molecule (Smac037) in green. Legend: black dashed lines - hydrogen bonds; green solid lines - hydrophobic interactions.

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