RESEARCH ARTICLE

Reversal of Multidrug Resistance and Computational Studies of Pistagremic Acid Isolated from *Pistacia integerrima*

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Abstract

Pistagremic acid (PA) is a bioactive triterpenoid isolated from various parts of *Pistacia integerrima* plants. The aim of this research was to investigate PA for reversion of multidrug resistant (MDR) mediated by P-glycoprotein using rhodamine-123 exclusion study on a multidrug resistant human ABCB1 (ATP-binding cassette, sub-family B, member 1) gene-transfected mouse T-lymphoma cell line *in vitro*. Results were similar to those with verapamil as a positive control. Docking studies of PA and standard Rhodamine123 were carried out against a P-gp crystal structure which showed satisfactory results. Actually, PA cannot bind exactly where co-crystallized ligand of P-gp is already present. However, the docking study predicted that if a compound gives a lesser score then it may have some potency. The docking scores of PA and Rhodamine were similar. Therefore, we can conclude that there are certain important chemical features of PA which are responsible for the inhibiting potency of P-gp.

Keywords: *Pistacia integerrima* - pistagremic acid - reversal of MDR in mouse lymphoma cells activities - molecular

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Introduction

Cancer is one of the leading causes of morbidity and mortality throughout the world. But the treatment of cancer is limited due to the development of multidrug-resistance (MDR) phenotype (Ferlay et al., 2015; Ferreira et al., 2015). One of the MDR-related mechanisms consequences from efflux pumps related to the family of ATP-binding cassette (ABC) proteins(Gottesman, 2002; Jin et al., 2012).

Among them, the P-glycoprotein (P-gp) belongs to the ABC superfamily of transporters. Members of this family are broadly divided into three groups. These proteins play a vital function in the in the MDR of pathogen yeasts and bacteria against drugs(Horio et al., 1988). The P-gp is mostly targeted for the treatment of cancer and other human diseases, P-gp is probably the best known of the ABC proteins and thus, it can be considered as a paradigmatic model for this family of transporters (Jones and George, 2004). The P-gp is long poly peptide protein has 1280 amino acids residues which are arranged as a single chain, with two homologous halves having 43% amino acid identity. A linker region of ~60 amino acids connects the two halves of the protein. Each half has six transmembrane domains (TM) and a hydrophilic domain containing an ATP-binding site, known as nucleotide binding domain (NBD) (Jara et al., 2013).

The primary binding region for Rhodamine123 in mouse P-gp was found to be a hydrophobic pocket involving binding site residues such as Ser218, Phe299, Val334, Leu335, Phe339, which have already been proposed to play a crucial role for substrate binding on experimental bases (Li et al., 2010).

Pistacia integerrima belongs to family *Anacardiaceae*. It is commonly known as kakar singhi. It is distributed in the eastern Himalayan range from Indus to Kumaon (Anonymous, (1998)) at a height of 12000 to 8000 feet. It is a medium sized deciduous tree that can achieve a height of forty feet. P. integrrima is a significant medicinal plant and is used as anti-inflammatory, antidiabetic agent, a blood cleanser, a tonic for gastrointestinal disorders, as cough expectorant (Upadhye, 2010; Pant., 2010). In Pakistan, galls of P. integrrima are used for the treatment of hepatitis and other liver disorders (Ahmad., 2010). It has also been reported to possess CNS depressant activity (Ansari., 1994a). The aim of current study was to isolated (PA) from *Pistacia integerrima* (Rauf et al., 2014a; Rauf et al., 2014b) and screen for multidrug resistance-reversal

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effects.

Materials and Methods

Experimental

Plant material

P. integrrima plant material was collected from Toormang, Razagram area of district Dir, Khyber Pukhtun Khawa province of Pakistan in the month of February 2010. The plant material was identified by Prof. Dr. Abdur Rashid of the department of Botany, University of Peshawar, Pakistan. A voucher specimen no (Bot.20037 (PUP) was deposited in the herbarium of the mentioned department.

Extraction and isolation

Shade dried and crushed bark of Pistacia integerrima (Stewart) (14 kg) was subjected to cold extraction with MeOH. MeOH extract (600g) was suspended in water and successively partitioned with hexane, CHCl3, EtOAc and BuOH. EtOAC fraction (30g) was subjected to Column chromatography on silica gel (Merck Silica gel 60 (0.063-0.200mm), 5 × 60 cm). The column was first eluted with hexane-Acetone (100:0 → 0:100) as a solvent system. A total of 33 fractions, RF-1 to RF-33 were obtained based on TLC profiles. Fraction RF-20 obtained at (100:0 → 15:100: Hexane-Acetone) gradient contained colorless crystals of various sizes and was separated from the solution by decantation. The crystals were washed with n- hexane for several times. To obtain pure and larger crystals, these crystals were re-grown from a mixture of hexane-acetone and chloroform (70:20:10) and thus obtained a compound named Pistagremic acid (50 mg). . Based on the above arguments and single crystal x-rays crystallographic studies, the structure of 1 was assigned as 2-Methyl-6-(4,4,10,13,14-pentamethyl-3-O-2,3,4,5,6,7,10,11,12,13,14,15,16,-17-tetradecahydro-1H-cyclopenta[a]-phenanthren-17-yl)hept-2-enoic acid (Arfan et al., 2011).

Assay for reversal of MDR in mouse lymphoma cells

The L5178 MDR and L5178Y parent cell lines were grown in McCoy`s 5A medium containing 10% heat-inactivated horse serum, was completed with L-glutamine and antibiotics. The cells were adjusted to a density of $2\times10^6$ mL resuspended in serum-free McCoy`s 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compound was added at 4 µg/ml final concentrations, and the samples were incubated for 10 minutes at room temperature. Verapamil was applied as a positive control [Cornwell MM, Pastan I and Gottesman MM: Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein in 10 μg/ml concentration.

Next, 10 μL (5.2 μM final concentration) of the indicator rhodamine 123 (Sigma, St Louis, MO, USA) was added to the samples and the cells were incubated for a further 20 minutes at 37ºC, washed twice and resuspended in 0.5 mL PBS for analysis. The fluorescence of the cell population was measured with a Partec CyFlow flow cytometer (Münster, Germany). The tested compound was dissolved in DMSO, which was also used as a solvent control. The percentage of mean fluorescence intensity was calculated for the treated MDR and parental cell lines as compared with the untreated cells. The activity ratio R was calculated via the following equation [Cornwell MM, Pastan I and Gottesman MM: Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J of Biol Chem. 262: 2166-2170, 1987.] on the basis of the measured fluorescence values:

$$FAR=\frac{MDR_{treated}/MDR_{control}}{parental_{treated}/MDR_{control}}$$

Molecular docking studies

The crystal structure of mice P-glycoprotein (P-gp) (PDB code= 4Q9L resolution 3.80Å) was obtained from protein data bank (PDB) (Berman et al., 2000). The structure was subjected to the energy refinement by swiss PDB viewer v4.1.0 program (Guex and Peitsch, 1997). The ligands structure ligands were through Chem sketch (Li et al., 2004) and Avogadro,s software (Hanwell et al., 2012).

The docking of Pistagremic acid and standard Rhodamine123 were carried out through Autodock Vina (Trott and Olson, 2010) and i-GEMDOCKv 2.1 software's (Hsu et al., 2011). The docking method was optimized by an already co-crystallized ligand of the P-gp receptor.

Furthermore, all the default parameters were used for both Autodock Vina and i-GEMDOCKv 2.1 software's (Rauf et al., 2015a; Rauf et al., 2015b; Rauf et al., 2015c). The docking analysis was carried out through LIGPLOT+ version v.1.4.5 (Wallace et al., 1995), PyMOL (DeLano, 2002) and Discovery studio visualizer softwares (Visualizer, 2005).

Results and Discussion

Shade dried and crushed plant materials of *P. integerrima* were repeatedly extracted with MeOH (X5) at room temperature. The extracts were suspended in water and successively partitioned with the various organic solvent to obtained CHCl3, EtOAc fractions. The EtOAc fraction (30g) was subjected to repeated chromatography using silica gel and further purification by recrystallization process. To obtain pure and larger crystals, these crystals were re-grown from a mixture of n-hexane-acetone and chloroform (70:30) and thus obtained a compound named Pistagremic acid (50 mg).

Reversal of Multidrug Resistance Mouse Lymphoma cells

On the basis of their previous anti-cancer potencies (Rauf et al., 2013), PA was selected for flow-cytometric cell cycle screening at the concentration (20 µg/ml). The effect in multidrug resistant mouse lymphoma cells is displayed in Table 1.

The fluorescence activity ratio (FAR) value was used to evaluate the ABCB1 transporter modulating potential. The values of SSC (side scatter count) and FSC (forward scatter count) were increased in the flow cytometry which

Table 1. Effects of PA on Rhodamine123 Accumulation in L5178 MDR Mouse Lymphoma Cells

	Sample	(final concentration) µg/ml	FSC	cv	Mean	FAR
1	PAR	-	2181	796	71.6	-
2	PAR	-	1910	597	65.8	-
3	MDR	-	2074	839	0.964	-
	MDR MEAN	-	2043	v	0.79	-
4	Verapamil	10	2012	769	3.71	4.69
5	Pistagremic acid	20	1436	1816	9.7	12.27
6	DMSO	0.20%	1494	955	n.d.	n.d.
7	MDR	-	2012	806	0.621	-

Table 2. Docking Scores of Pistagremic acid and Standard Rhodamine123 against Mouse P-glycoprotein

Ligands	Autodock Vina	i-GEM DOCK		
	B. Affinity	Total Energy	VDW	HBond
Pistagremic acid	-7.4	-72	-71	-1
Rhodamine123	-8.2	-87	-86	-1

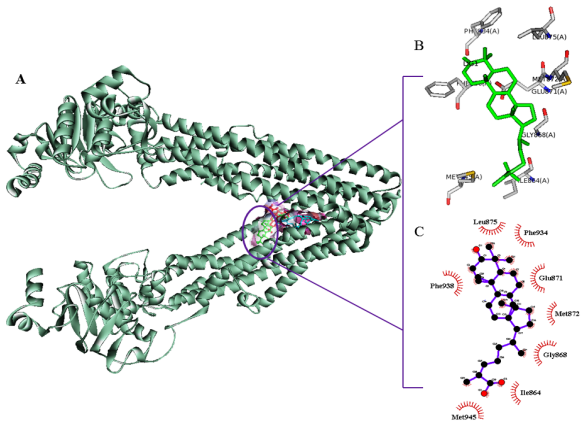

Figure 1. The Predicted Docked Poses of Pistagremic Acid (PA). In the above figure "A" the encircle line represent sdocked PA shown by green colors ball and sticks while the standard Rhodamine123 is shown by the red color ball and sticks. The co-crystallized ligand in the binding pocket of P-gp is observed from the cyan color sticks. The "B" shows the 3-D schematic representation of the docked Pistagremic acid while the "C" shows the 2-D interactions of important residues in the binding pocket of Pistagremic acid

showed that the compounds (PA) had membrane effect and the granulation of cytoplasm was increased. The FAR values obtained indicated that PA is very effective MDR modulator in a short time experiment. Verapamil, which is a calcium channel blocker and chemo sensitizer, was used as a positive control. On MDR mouse lymphoma cells the PA was screen in one concentration (20 µg/ml). The PA was strong modulators of the efflux-pump activity (FAR 12.27, 20 μg/ml) (Table 1).

Molecular docking studies have important applications in the field of drug discovery. It initially recognized the inhibiting potency of new compounds against the targeted receptors. Our molecular docking studies can co-relate with the in-vitro results of Pistagremic acid. The docking studies of Pistagremic acid and standard Rhodamine123 was carried out against the crystal structure of P-gp. The docking of Pistagremic acid gives very satisfactory results as it is confirmed from the docking Table 1 and from Figure 1. Actually, Pistagremic acid cannot bind exactly where already co-crystallized ligand of P-gp is present. Generally, docking study predicts that, if a compound gives lesser interaction energy then that compound has higher activity. Hence the docking scores of Pistagremic acid are near to the Rhodamine (Table 2). Therefore, we can conclude that there are certain important structural features of Pistagremic acid which are responsible for the inhibiting potency of P-gp from mice.

The detailed interactions (Figure 1) of the Pistagremic acid against the P-gp shows us that there is a total of eight hydrophobic contacts observed from the residues i.e Ile864, Gly868, Glu871, Met872, Leu875, Phe934, Phe938 and Met945. But no hydrogen bond interaction has been observed from any residues in the binding pocket of P-gp. So this may be the reason for such a satisfactory result of Pistagremic acid as comparatively with the standard Rhodamine.

In conclusion, it is concluded that there are certain important structural features of PA which are responsible for the inhibiting potency of P-gp from mice. The docking scores of PA and Rhodamine are similar. This study directed the researcher to isolate PA and synthesize their derivatives and to enhance their anticancer potential.

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References

Ahmad N, Waheed A, Farman M, Qayyum A (2010). Analgesic and anti-imflammatory effects of *Pistacia integrrima* extracts in mice. *J Ethnopharmacol*, **129**, 250.

Anonymous (1998). The Wealth of India. A Dictionary of Indian raw materials and industrial products, publication and Information Directorate, CSIR, New Delhi, **8**.

Ansari SH, Ali M, Qadry JS, Siddiqui N (1994a). Update Ayurveda, **94**.

Arfan M, Rauf A, Tahir MN, et al (2011). 2-Methyl-6-(4, 4, 10,

13, 14-pentamethyl-3-oxo-2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydro-1H-cyclopenta [a] phenanthren-17-yl) hept-2-enoic acid. *Acta Cryst*, **67**, 711.

Berman HM, Westbrook J, Feng Z, et al (2000). The protein data bank. *Nucleic Acids Res*, **28**, 235-42.

DeLano WL (2002). PyMOL. DeLano Scientific, *San Carlos, CA*, **700**.

Ferlay J, Soerjomataram I, Dikshit R, et al (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*, **136**, 359-86.

Ferreira RJ, dos Santos DJ, Ferreira M-JU (2015). P-glycoprotein and membrane roles in multidrug resistance. *Future Med Chem*, **7**, 929-46.

Gottesman MM (2002). Mechanisms of cancer drug resistance. *Annual Review Med*, **53**, 615-27.

Guex N, Peitsch MC (1997). SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modeling. *Electrophoresis*, **18**, 2714-23.

Hanwell MD, Curtis DE, Lonie DC, et al (2012). Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *J Cheminformatics*, **4**, 17.

Horio M, Gottesman MM, Pastan I (1988). ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proceedings National Academy Sci*, **85**, 3580-4.

Hsu KC, Chen YF, Lin SR, et al (2011). iGEMDOCK: a graphical environment of enhancing GEMDOCK using pharmacological interactions and post-screening analysis. BMC bioinformatics, 12, S33.

Jara GE, Vera DMA, Pierini AB (2013). Binding of modulators to mouse and human multidrug resistance P-glycoprotein. A computational study. *J Molecular Graphics Modelling*, **46**, 10-21.

Jin MS, Oldham ML, Zhang Q, et al (2012). Crystal structure of the multidrug transporter P-glycoprotein from Caenorhabditis elegans. *Nature*, **490**, 566-9.

Jones P, George A (2004). The ABC transporter structure and mechanism: perspectives on recent research. *Cellular Molecular Life Sci CMLS*, **61**, 682-99.

Li Y, Yuan H, Yang K, et al (2010). The structure and functions of P-glycoprotein. *Current Med Chem*, **17**, 786-800.

Li Z, Wan H, Shi Y, et al (2004). Personal experience with four kinds of chemical structure drawing software: review on chem draw, chem window, isis/draw, and chem sketch. *J Chemical Informat Computer Sci*, **44**, 1886-90.

Pant. s, samant s.s (2010). Ethanobotanical observation in the Momaula Reserve Forest of Koumoun, West Himalaya, India. *Ethanobotanical Leaflets*, **1493**.

Rauf A, Khan R, Raza M, et al (2015a). Suppression of inflammatory response by chrysin, a flavone isolated from Potentilla evestita Th. Wolf. In silico predictive study on its mechanistic effect. *Fitoterapia*, **103**, 129-35.

Rauf A, Saleem M, Uddin G, et al (2015b). Phosphodiesterase-1 Inhibitory Activity of Two Flavonoids Isolated from Pistacia integerrima JL Stewart Galls. *Evidence-Based Complementary Alternative Med*, **10**, 506564.

Rauf A, Uddin G, Khan H, et al (2015c). Anti-tumour-promoting and thermal-induced protein denaturation inhibitory activities of β-sitosterol and lupeol isolated from Diospyros lotus L. *Natural Product Res*, 1-3.

Rauf A, Uddin G, Latif A, et al (2014a). Pistagremic Acid, a novel antimicrobial and antioxidant isolated from Pistacia integerrima. *Chemistry Natural Compounds*, **50**, 97-9.

Rauf A, Uddin G, Siddiqui BS, et al (2014b). In-vivo antinociceptive, anti-inflammatory and antipyretic activity of pistagremic acid isolated from Pistacia integerrima. *Phytomedicine*, **21**, 1509-15.

Trott O, Olson AJ (2010). Auto Dock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Computat Chem*, **31**, 455-61.

Upadhye ASAAR (2010). Pharmacogonostic and phytochemical evaluation of leaf galls of Kakadshringi used in Indian system of medicine. *J Scientific Industrial Res*, **69**.

Visualizer DS (2005). Accelrys Software Inc. *Discovery Studio Visualizer*, **2**.

Wallace AC, Laskowski RA, Thornton JM (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Enginee*, **8**, 127-34.