SUPPLEMENTARY MATERIAL

Anti-melanoma and UV-B protective effect of microbial pigment produced by marine *Pseudomonas aeruginosa* GS-33

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Abstract

Bioactivity of a microbial pigment, extracted from fermented broth of culture marine *Pseudomonas aeruginosa* was screened for anticancer activity against human skin melanoma cell line SK-MEL-2. Upon characterisation the pigment was confirmed as Phenazine-1-carboxylic acid (PCA). The PCA was found effective against SK-MEL-2 cell line at low concentration (GI₅₀ value <10 µg/mL). Reduced cell density and cell shrinkage with typical morphological changes such as rounding of cells with loss/breaking of cell membrane were seen in SK-MEL-2 cells treated with PCA and Adriamycin. The pigment exhibited UV-B protecting activity as calculated by *in-vitro* spectrophotometric assay and potentiated sun protection factor of commercial sunscreen lotion. Moreover; the pigment was nontoxic up to concentration of 100 ppm as assessed erythrocyte haemolysis assay. These results suggest that microbial pigment PCA could be effective and promising in the treatment as well as prevention of melanoma skin cancers.

Keywords: microbial pigment; phenazine-1-carboxylic acid; *Pseudomonas aeruginosa*; anti-skin melanoma; UV-B protection; cytotoxicity

Experimental

Source of bacterial isolate

The bacterial strain GS-33 used in the present studies was already isolated from marine water and identified as *Pseudomonas aeruginosa* on the basis of its morphological, physiological, biochemical and molecular characterisation. The isolate was capable of producing antifungal compound PCA (data communicated elsewhere).

Large scale production, purification and characterisation of PCA

For large scale production of phenazine pigments, about 2% of 18 hours grown culture was inoculated in 5 L Erlenmeyer flask containing 2 L pigment production medium (g/L: peptone 20, glycerol 20, NaCl 5, KNO₃ 1, pH 7.2) and incubated for 48 h at 28 °C at 120 rpm. Biomass was separated from culture broth by centrifugation at 10000 rpm for 10 min. Further purification of PCA was achieved by following the methods reported by Rane et al. (2007) with some modifications. In brief, phenazine rich cell free culture supernatant was passed through XAD-4 column (2.5 cm diameter, 30 cm height) with adsorption rate of 2 bed-volumes per hour. The column was washed with 500 ml distilled water followed by washing with 50 ml of 30 % methanol at the rate of 10 bed-volumes per hour. Adsorbed phenazines were eluted with 70% methanol at the rate of 1 bed volume per hour. Elute was acidified and extracted thrice with equal volume of benzene to separate PCA. Then, benzene layer containing PCA was washed thrice with equal volumes distilled water to remove water soluble impurities and further concentrated on rotary vacuum evaporator. Concentrated PCA solution was loaded on top of silica gel column (120-200 gel mesh size, 2.5 cm diameter, 30 cm height) equilibrated with benzene. After passing 25 ml of pure benzene, PCA was eluted with benzene: acetic acid (19:1) and fractions having 367 nm as λ_{max} were pooled together, concentrated and subjected to crystallization overnight. Purified crystals (Lemon yellow colored) were obtained as shown in Figure S1 and subjected to further chemical characterisation.

Characterisation of purified phenazine compounds

Chemical characterisation of purified phenazine was accomplished by using spectroscopic as well as chromatographic analysis techniques. UV-Vis spectrum of purified phenazine dissolved in methanol was recorded with UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). FTIR analysis was performed to determine functional groups of purified phenazine by FTIR spectrometer (Spectrum Two, FTIR-88522, Perkin Elmer, USA) using KBr pellet method. Thin layer chromatography of purified phenazine was performed using aluminium plates precoated with silica gel (60-F254) of 0.2 mm thickness (Merck, Mumbai, India) and solvent system

benzene-acetic acid (95:5) which was visualized at 254 nm and R_f value was determined (Rosales et al. 1995). Further, characterisation of purified phenazine was carried out by analytical HPLC (Shimadzu LC-8A) using a C_{18} reversed-phase column, eluted with solvent system acetonitrile-water (70:30) at flow rate 1 mL/min and detected by UV-Vis detector at 248 nm as reported previously (Liu et al. 2006). MS/MS was used with positive mode electro spray ionization (ESI) mode (500 MS, Varian Inc, USA) to produce mass spectra using a scan range from 100 to 500 (m/z) with a scan time of 20 min. Data obtained was compared with information available in literature to confirm purified compound to be PCA.

In-vitro anticancer activity of PCA

The anticancer activity of PCA was studied by using sulforhodamine B (SRB) assay (Skehn et al. 1990). The human skin melanoma cell line (SK-MEL-2) cell line was procured from National Centre for Cell Science (NCCS), Pune, India then maintained and grown in ideal laboratory conditions using RPMI 1640 medium containing 10 % fetal bovine serum and 2 mM L-glutamine. In order to screen for anticancer potential of PCA, cells were inoculated into 96 well microtiter plates (90 μ L/well) at appropriate plating density. After cell inoculation, the microtiter plates were incubated at 37°C, in 5% CO₂, 95% air and 100 % relative humidity. After 24 h of incubation the cell population at the time of drug addition (Tz) was determined by fixing cells *in situ* with trichloroacetic acid (TCA) in microtiter plate labelled as time zero.

Then PCA and Adriamycin (Doxorubicin- standard positive control anticancer drug) were solubilized in DMSO to prepare concentrated stock solutions which were stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and 10 μ L of appropriately diluted solutions were added to the 90 μ L of cell suspension present in appropriate microtiter wells, resulting in the required final drug concentrations of 10, 20, 40 and 80 μ g/ml. After compound addition, plates were incubated at standard conditions for 48 h and assay was terminated by the gentle addition of 50 μ L of cold TCA (30 % w/v) and incubated for 60 min at 4°C. Then after discarding supernatant; the plates were washed five times with tap water and air dried at room temperature. The 50 μ L of 0.4 % SRB solution in 1 % acetic acid (w/v) was added to each of the wells and plates were incubated at room temperature for 20 minutes. After staining, unbound and the residual dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently eluted with Trizma base (10 mM), and the absorbance was read at 540 nm using Elisa Plate Reader.

Using absorbance measurements; Tz (time zero), C (control growth), and Ti (test growth in the presence of drug at different concentration levels); the percentage control growth at each of the drug concentration levels was calculated.

Percentage growth inhibition was calculated as follow;

$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations where } Ti \geq Tz \text{ and}$$

$$[(Ti-Tz)/Tz] \times 100 \text{ for concentrations where } Ti < Tz.$$

The dose response parameters were calculated for both PCA and Adriamycin. The 50% of growth inhibition (GI50) was calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$. Indicating the measure of drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the incubation in presence of drug.

The drug concentration resulting in total growth inhibition (TGI) was calculated from $Ti = Tz$. The concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning (LC50) indicating a net loss of cells following treatment was calculated from $[(Ti-Tz)/Tz] \times 100 = -50$.

The values of these three dose response parameters were calculated if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were expressed as greater or less than the maximum or minimum concentration tested.

These cytotoxicity studies were performed at Tata Memorial Centre, Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai, India.

In vitro determination of UVB protection by sun protection factor (SPF)

In vitro spectrophotometric method was used for SPF determination (Dutra et al., 2004; Borase et al., 2014). In brief, commercially available sunscreen with labelled SPF of 15 was constituted as control respectively while, test contains the above commercial sunscreens spiked with PCA at concentration of 0.1, 0.2 and 0.4 % w/w of sunscreen. One gram each from control and test sunscreen formulations was weighed separately and added in 100 ml volumetric flask and were diluted to 100 ml with ethanol, stirred for 5 min and filtered. Initial 10 ml filtrate was discarded and 5 ml filtrate was diluted to 50 ml with ethanol. Then 5 mL of the diluted solution added in to the volumetric flask and volume made up to 25 mL using ethanol. Absorbance of all control and test formulations was measured between wavelengths 290 to 320 nm at interval of 5 nm. Experiments were performed in triplicate and values were reported as mean $\pm$ standard deviation. SPF of test and control sunscreen preparations were calculated by the following equation,

$$SPF = CF \times \sum EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where, CF - correlation factor =10, Σ - Sum of $EE(\lambda) \times I(\lambda) \times Abs(\lambda)$ for absorbance from 290 to 320 nm, $EE(\lambda)$ is the erythermal effects spectrum, $I(\lambda)$ - solar intensity spectrum, value of EE

$(\lambda) \times I(\lambda)$ is constant and was determined by Sayre et al., 1979 as given in Table S1, Abs (λ) - Abs- absorbance of sunscreen product.

Table S1: The normalized product function used in the calculation of SPF data

$\lambda(\text{nm})$	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
	=1.000

EE: erythral efficiency spectrum; I : solar simulator intensity spectrum (Sayre et al., 1979).

Percentage fold increases of individual sunscreen were calculated using the formula

$$\text{Percentage fold increase} = \frac{(b - a)}{(a)} \times 100$$

where a-SPF of sunscreen lotion and b-SPF of sunscreen fortified with PCA.

Toxicity analysis of PCA by erythrocyte haemolysis assay

Cytotoxicity of PCA towards normal human cells was evaluated by RBCs haemolysis assay (Kumar et al. 2011). Blood from healthy volunteers was collected with a sterile syringe. To inhibit the coagulation of blood, 0.5 mL of 0.3 % EDTA solution was mixed to 9.5 mL of the blood. It was then centrifuged at 1000 rpm for 10 min to remove the supernatant containing platelets and plasma. The pellet containing RBC was re-suspended in 10 mL of phosphate buffer saline (PBS) having pH 7.4 and the process was repeated three times. Finally, the cells were suspended in PBS so as to get a uniform cell suspension having 5 % (v/v) density. The 2 mL of erythrocyte suspension was added to each tube. The appropriate aliquots of PCA solution from stock prepared in PBS was added in different tubes so as to get desired final concentrations. The tubes were inverted and gently shaken to maintain contact of the erythrocytes with the PCA and incubated at 37 °C for 1 h. The sterile deionized water was used as a positive control while PBS buffer acted as a negative control. After incubation, the samples were centrifuged at 3000 rpm for 5 min to pellet out the RBCs. The absorption of the

supernatant was measured at 540 nm against appropriate blank. The percent haemolysis relative to positive control was calculated for each sample using following formula,

$$\text{Haemolysis (\%)} = \frac{(OD \text{ test sample} - OD \text{ negative control})}{(OD \text{ positive control} - OD \text{ negative control})} \times 100$$

Where OD is the optical density measured at 540 nm.

Figure S1: Crystal of purified Phenazine-1-carboxylic acid observed at 40x

Figure S2: UV-Vis spectrum of purified Phenazine-1-carboxylic acid with sharp absorption peaks at 251 and 367 nm

Figure S3: FTIR spectrum of purified Phenazine-1-carboxylic acid exhibiting the band at 3411 cm^{-1} (OH group), an overtone at 2642 cm^{-1} (COOH group) and intense peak at 1739 cm^{-1} (C=O) indicating presence of carboxylic group. Peaks were also exhibited in the region 1600 and 1400 cm^{-1} for C=C (stretch in ring) aromatic. Peaks at 1470 and 1264 cm^{-1} represented aromatic CH bends. The peaks at 3063 , 992 - 670 cm^{-1} were also exhibited due to C-H of aromatic in molecule. All these FTIR peaks matched with the previously reported compound PCA (Brisbane et al. 1987).

Figure S4: TLC chromatogram of purified Phenazine-1-carboxylic acid (R_f value 0.5294)

Figure S5: HPLC chromatogram of purified Phenazine-1-carboxylic acid with retention time of 1.970 min.

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Figure S6: MS-MS spectrum of purified Phenazine-1-carboxylic acid

Table S2: Dose response parameter calculated from graph for Human skin melanoma cell line (SK-MEL-2) treated with Phenazine-1-carboxylic acid and Adriamycin

Dose response parameter calculated from graph for Human skin melanoma cell line (SK-MEL-2)			
Parameter (→) Compounds (↓)	LC50	TGI	GI50
Phenazine-1-carboxylic acid	>80	>80	<10
Adriamycin (Doxorubicin)	<10	<10	<10

GI50: Growth inhibition of 50 %; TGI: Drug concentration resulting in total growth inhibition;
LC50: Concentration of drug resulting in a net loss of 50% cells following treatment

Table S3: SPF of commercial sunscreen lotion, PCA and their mix formulations

PCA		SPF of sunscreens lotion after addition of PCA	
Concentration used	SPF	SPF 15	SPF 30
Control (0 ppm)	-	14.70 ± 0.36 ^a	29.24 ± 0.41 ^a
25 ppm	1.43 ± 0.08 ^a	16.20 ± 0.38 ^b (10.21)	31.97 ± 0.34 ^b (9.33)
50 ppm	2.55 ± 0.07 ^b	17.45 ± 0.50 ^c (18.66)	34.12 ± 0.34 ^c (16.80)
100 ppm	4.73 ± 0.14 ^c	19.84 ± 0.67 ^d (34.94)	35.08 ± 0.34 ^d (25.65)

Results are expressed as mean ± standard deviation (n=3). Numerical values within a column followed by a different superscript letter are significantly different from each other according to Fisher's least significant difference test ($p \leq 0.05$). Values in parenthesis indicate percentage fold increase in SPF of commercial sunscreen lotion after addition of PCA.

Figure S7: Toxicity analysis of PCA by erythrocyte haemolysis assay, DW-distilled water as positive control showing complete haemolysis; PS-physiological saline as negative control with no haemolysis; PCA in ppm (25, 50 and 100) test samples

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