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Design and synthesis of phosphonate functionalized naphthalenediimide: Application to induce mitochondria mediated apoptosis in the human skin melanoma cells (SKMEL2)

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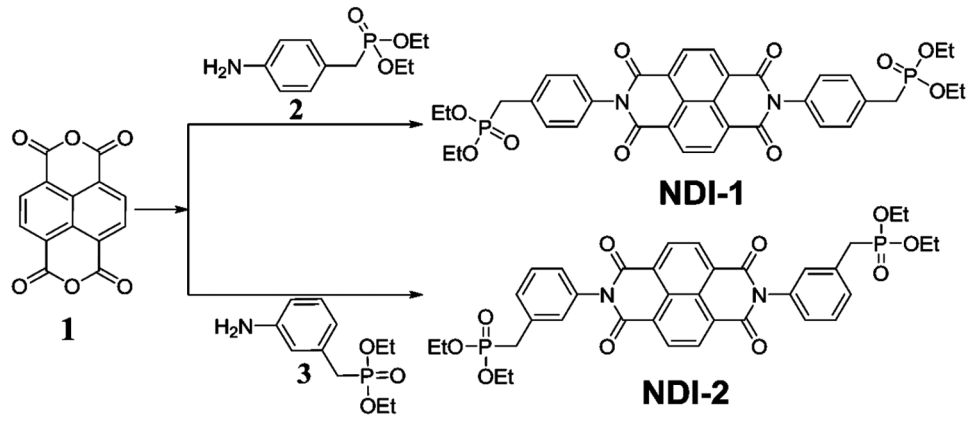
New phosphonate functionalized naphthalenediimides (**NDI-1** and **NDI-2**) have been synthesized and evaluated as potential drug candidates to treat the skin malignancy. The aforementioned candidates have resulted in an induced apoptosis pathway of cytotoxicity, at concentrations lower than 4 μM, in human skin melanoma cells, and elicited a stronger apoptotic response in cells. Regulation of the Bcl2, Caspases, Bak1, Bax protein in NDI-induced apoptosis in cells has been analyzed by real time PCR, and the cell viability and apoptosis determined by flow cytometry. The obtained results indicate that NDI-induced apoptosis by regulation of Bcl2 and Bak1 occurs in human skin melanoma cells (SKMEL-2).

Keywords: Phosphonate, Naphthalenediimide, Mitochondria, Apoptosis, SKMEL2

Melanocytes are the skin cells that become malignant and form malignant melanoma. The drug resistance developed against the current chemotherapy drugs and the extremely aggressive behaviour results in high mortality[1]. The poor survival rates in malignant melanoma patients were also due to slow development in promising therapeutic molecules[2]. The search for effective anti-melanocyte molecules has become an important priority due to limited success of surgical therapy in treating metastatic melanoma. Single molecules or blended chemotherapy radiotherapy was not successful in controlling the melanocytes migration[2-5]. The failure of chemo- and radio therapeutic treatments suggest the presence of intrinsic resistance mechanism. The drug resistance is due to rapid mutations in the p53 gene in skin melanoma cells that develop low or no response to the radiotherapy, therapeutic antibodies, and multi-component therapy[6].

The aromatic imides are reported as promising antimelanoma pharmacophores[7]. Amongst this group, naphthalene diimides (NDIs) are a class of therapeutic molecules[8,9]. In a recent report, a series of substituted NDI derivatives with positively charged functional groups were prepared, which selectively inhibited the growth of malignant cells at sub-micromolar concentration[10]. Furthermore, *tetra*-core-substituted NDI ligands for telomere uncapping with DNA (Deoxyribonucleic acid) damage, inhibition of expression of oncogenes, genomic and chromosomal instability by telomere aggregate formation in cancer cells have been also studied in detail[11]. Both these reports showed promising inhibitory effect through telomeric quadruplex thermal stabilization by employing NDI derivatives. It is possible that quadruplex binding is favoured by the electron deficient NDI aromatic core having a planar surface.

In this study, we have investigated two new NDIs, **NDI-1** and **NDI-2** derivatives bearing phosphonate ester functionalities (Scheme 1) to induce apoptosis in human skin melanoma cells (SKMEL2). In addition, we have investigated a hypothesis- whether the novel derivatives of NDI can induce irreversible apoptosis in chemo-resistant skin melanoma cells. The role of



Scheme 1 — Synthetic route of **NDI**-1 and **NDI-2**

caspases and Bcl-2 family proteins in apoptosis induction by NDI compounds were analysed. The apoptosis induction in SKMEL2 cells, pro-apoptotic and anti-apoptotic gene expression with **NDI-1** and **NDI-2** were analysed by qPCR. The cell cycle inhibition in the melanoma cells and the active caspases also confirm the irreversible pathway of apoptosis in skin melanoma cells. This helps us to warrant that **NDI-1** and **NDI-2** derivatives are effective agents for induction of apoptosis in skin cancer cells of human.

Experimental Section

General Methods and Materials

All reagents were purchased either from Sigma Aldrich Chemical, Merck, Life Technol. Inc., USA, Promega Inc. USA, Biorad Inc. USA, and were used without further purification. All solvents were received from commercial sources and purified by standard methods. Melting points were determined in open glass capillaries and are uncorrected. ^{1}H NMR spectra were recorded on an ADVANCE 300 MHz or INNOVA 500 MHz NMR spectrometer and are referenced to TMS. Mass spectrometric data were obtained by electron spray ionization (ESI-MS) on an Agilent Technologies 1100 Series (Agilent Chemistation Software) mass spectrometer. High-resolution mass spectra (HRMS) were obtained on an ESIQTOF mass spectrometer. FTIR spectra were obtained on a Perkin-Elmer Spectrum-100 spectrometer. HPLC analysis of **NDI-1** and **NDI-2** was done by using Agilent Prostar HPLC (Fig S1 supporting information).

The aniline substituted phosphonate esters **2-5** were prepared according to methods reported previously[12-14].

Synthesis and Characterization

General procedure for the synthesis of phosphonate naphthalenediimides, NDI-1 and NDI-2

A phosphonate ester (**4** and **5**) (14.6 mmol) and 1,4,5,8-naphthalene tetracarboxylic dianhydride **3** (1.61 g, 6.0 mmol) were suspended in 20 mL of *N*,*N'*-dimethylacetamide (DMA) under a nitrogen atmosphere. The mixture was heated to 120°C with vigorously stirring for 12 h. The reaction mixture was cooled to RT and poured into 100 mL of cooled 1N aqueous HCl. The resulting suspension was extracted with dichloromethane (2×100 mL) and the organic layer was separated. The combined organic phases were washed with brine solution (2×50 mL), dried over anhydrous $MgSO_4$ and the solvent evaporated using a rotary evaporator. The obtained crude product was purified by column chromatography (silica gel 100-200, $CHCl_3$: MeOH, 9:2, *v/v* ratio) followed by recrystallized from $CHCl_3$: hexane to give a solid product.

NDI-1: Pale yellow solid. Yield 72%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.27 (t, J = 6.9 Hz, 12 H), 3.23 (d, J_{HP} = 21.9 Hz, 4H) 4.04- 4.13 (m, 8H), 7.28 (d, J = 8.1 Hz, 4H), 7.51 (d, J = 8.4 Hz, 4H), 8.84 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.37, 32.63, 34.47, 62.31, 126.88, 127.10, 128.55, 130.91, 131.35, 132.97, 133.21, 162.79; IR (KBr): 769, 1020, 1237, 1248, 1346, 1523, 1580, 1673, 1713, 2966, and 2985 cm^{-1}; ESI-MS: m/z (%) 720 (100) $[M + 2H]^+$; HRMS: Calcd for $C_{36}H_{37}O_{10}N_2P_2$: 719.1917. Found (ESI+) $[M + H]^+$: 719.1926.

NDI-2: Pale yellow solid. Yield 68%. m.p. 214-216°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.24 (t, J = 6.9 Hz, 12 H), 3.21 (d, J_{HP} = 21.9 Hz, 4H) 4.00-4.09 (m, 8H), 7.25- 7.33 (m, 2H), 7.32 (s, 2H), 7.50-

7.57 (m, 4H), 8.83 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 16.41, 32.75, 34.59, 62.48, 126.97, 129.84, 138.58, 131.36, 133.44, 134.70, 162.77; IR (KBr): 967, 1025, 1054, 1249, 1347, 1582, 1675, 1712, 2930, and 2982 cm^{-1}; ESI-MS: m/z (%) 720 (100) $[M + 2H]^+$; HRMS: Calcd for $C_{36}H_{37}O_{10}N_2P_2$: 719.1917. Found (ESI+) $[M + H]^+$: 719.1918.

Cell culture

Human skin melanoma cancer cells (SKMEL2 cells) were cultured in DMEM (Thermo. Inc., USA) supplemented with 2 mM glutamine and 10% (v/v) heat-inactivated FBS at 37°C in the presence of 5% CO_2. Unless otherwise stated, SKMEL2 cells were used in all the studies. All drug-resistant cells were maintained in drug-free media for at least 2 weeks before any experiment.

Determination of cell viability and the MIC

Cells were cultured to reach a density of 2×10^5 cells/mL in 96 well plates. Then the compounds **NDI-1** and **NDI-2** and control drug 5-FU were diluted and added to the wells respectively. The dilutions were prepared by using 0.1% *v/v* DMSO. The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Inc. USA) was used. Procedure followed were according to the manufacturer's instructions. The absorbance was recorded at 490 nm using a 96 well Multiscan Ascent (Thermo Inc. USA).

Expression of Pro and Anti-apoptotic genes

RNA transcripts were quantified by real-time PCR. The First strand of cDNA was mixed with SYBR Green PCR master mix (Biorad Inc. USA) and genes specific primers, and Real-time PCR was performed in triplicate for each sample according to the manufacturer's instructions (Biorad Inc. USA). For each single-well amplification reaction, a threshold cycle (CT) was observed in the exponential phase of amplification and the quantitation of relative expression levels was achieved using standard curves for both target and endogenous controls. The primers used are given in Table S1 (Supplementary Information).

Analysis of apoptosis

The cell density (2×10^5 cells /mL) were used for the assay. Apoptosis was induced by adding the compounds in the wells and analyzed by caspase activity detection and phosphatidylserine exposure analysis.

Poly caspases analysis by Flow cytometry

The Vybrant® FAM Poly Caspases Assay Kit (Thermo, Inc., USA) used to detect active caspases in apoptotic cells is based on a fluorescent inhibitor of caspases (FLICA™) methodology. The assay was performed on an Attune flow cytometer (Life Technol. Inc., USA) according to the manufacturer's instructions. The staining procedure followed the manufacturer's instructions (Life technol. Inc., USA) for Vybrant® FAM Poly Caspases Assay Kit (V35117). The Vybrant® FAM Poly Caspases Assay Kit provides FAM-VAD-FMK FLICA reagent, a generic probe for the detection of most caspases (including caspase -1, -3, -4, -5, -6, -7, -8, and -9); Hoechst 33342 stain; and propidium iodide stain. Cells labelled with Vybrant® FAM Poly Caspases Assay Kit can be analyzed by flow cytometry using 488 nm excitation and emission filters appropriate for Alexa Fluor® dye (FAM signal) and Texas Red® dye (propidium iodide signal), and using UV excitation and an emission filter appropriate for Hoechst 33342 dye.

Flow cytometry (Annexin V-FITC/ propidium iodide staining) of phosphatidylserine exposure

In presence of **NDI-1** and **NDI-2**, SKMEL2 cells were assayed for phosphatidyl serine exposure using the Annexin V-FITC Apoptosis Detection Kit-I (Life Technol. Inc., USA) according to the manufacturer's instructions. Stained samples were analyzed using an Attune flow cytometer (Life Technol. Inc., USA). Data were recorded using the Attune cytometric software for further analysis.

Analysis of cell cycle in SKMEL2 cells

In twelve-well plates (50,000 cells/well) of SKMEL2 cells were plated. After 24 h, **NDI-1** and **NDI-2** were added. The cells were washed twice with the PBS and fixed in 70% (*v/v*) ice-cold ethanol at 4°C for 24 h. 50 µL of RNase A solution (100 µg/mL in PBS) was added to the cells. The fixed cells were stained with propidium iodide (Life technologies Inc. USA). The samples were then analyzed in an Attune flow cytometer (Life technologies Inc. USA).

Statistical analysis

All values are expressed as mean ±SD. Statistical significance was compared between treatment groups and controls using Student's *t*-test. ***P-values*** < 0.05 were considered significant.

Results and Discussion

Synthesis of compounds

The phosphonate functionalised **NDI-1** and **NDI-2** derivatives were synthesised by reacting 1,4,5,8-naphthalenetetracarboxylic dianhydride with aniline-substituted phosphonate esters **2** and **3** in *N*,*N*′-dimethyl acetamide (DMA) at 120°C under a nitrogen atmosphere. **NDI-1** and **NDI-2** were obtained in good yields (Scheme 1).

Evaluation of anti-proliferation action of NDI-1 and NDI-2

Skin melanoma cells (SKMEL2) were used to evaluate the antiproliferative efficiency of the NDI compounds. The cell viability in treated Human melanoma cells was analyzed by employing the MTS assay. Firstly, cell lines were exposed with **NDI-1** and **NDI-2** and 5-fluorouracil (**5-FU**) for 24 h with varying concentrations (0 to 256 μM). The 5-FU, a drug commonly used to treat the skin melanomas and the cell viability was determined (Fig. 1). Fig. 1 shows mean IC_{50} values (50% growth inhibition) obtained from three independent experiments. The compounds **NDI-1** and **NDI-2** act in a time and dose dependent manner on the treated SKMEL2 cells and the viability was found decreased. The IC_{50} values of **NDI-1** and **NDI-2** were found to be 4 μM and 16 μM, respectively, while there was no significant differences obtained for 24 h and 48 h.

Analysis of membrane proteins of NDI treated SKMEL2 cells

For the analysis of effect of NDI on cancer cell population, flow cytometry of the phosphotidylserine on the membranes of the apoptotic cells was undertaken. The SKMEL2 cells were used for the study. The cells were incubated with various concentrations from 4 μM and 16 μM of **NDI-1**, **NDI-2** and **5-FU** for 24 h, respectively.

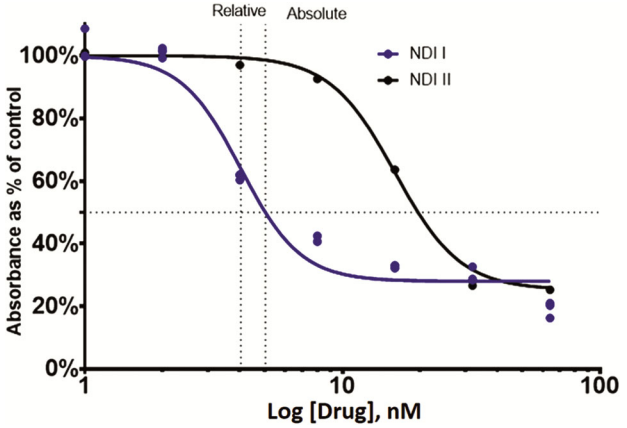


Fig. 1 — Effect of NDI compounds on viability of SKMEL2 cells. SKMEL2 cells after 24 h incubation at 37°C in the presence of 5% CO_2 (w/v). The compounds were added to the cells in DMEM medium. CC-50 values are the molar concentration resulting in 50% dead cells; the results are sum of the three independent experiments as compared to that of untreated cells. Error bars shows SD. set as 100%; n = 3; ****P<0.0001, ***P<0.001, **P<0.01, *P< 0 .05 and NS-not significant

Fig. 2 shows apoptotic cell population in NDI treated and untreated cells. The percentage of apoptotic cells in the SKMEL2 was found to be 36.2% in presence of **NDI-1** at 4 μM concentration. The **NDI-2** also induced apoptosis (42.2% cell population) in SKMEL2 cells. In contrast, 66.3% apoptotic cell population found in presence of 16 μM of the **5-FU** in SKMEL2 cells. The live cell population was decreased from 82.59% (untreated cells) to 63.6% and 56.6% in **NDI-1** and **NDI-2** treated cells, respectively.

Role of Caspases

Caspases are important in induction of apoptosis in cells[15]. The FAM polycaspase kit was used to analyze the caspase activation in the skin melanoma cells. Poly caspases (caspases 3, 8 and 9) were found active in the cells treated with **NDI-1** and **NDI-2** compounds (Fig. 3). The skin cancer cells were treated with 4 μM and 16 μM of **NDI-1**, **NDI-2** and **5-FU**, respectively. The activated caspases cell population was increased up to 6.5% and 5.8% in **NDI-1** and **NDI-2** treated cells, respectively. The activated caspases induce the

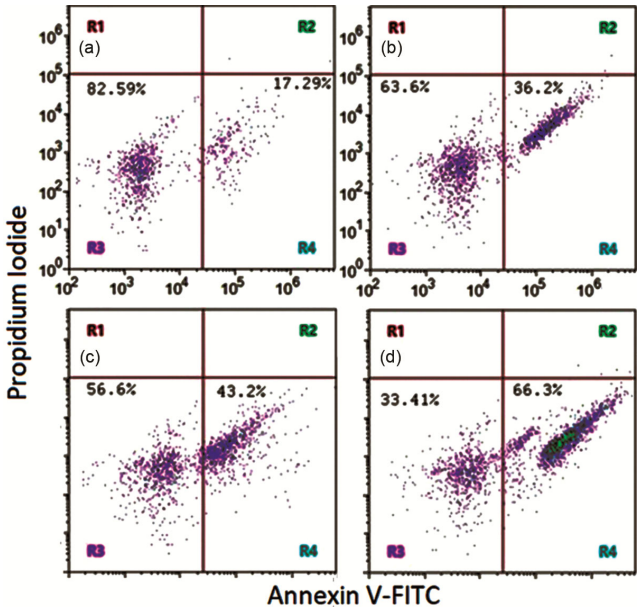


Fig. 2 — Analysis of exposure of phosphatidylserine on SKMEL2 cells by flow cytometry. (a) Untreated SKMEL2 cells, (b) **NDI-1** in SKMEL2 cells, (c) **NDI-2** in SKMEL2 cells and **(d) 5-FU** in SKMEL2 cells

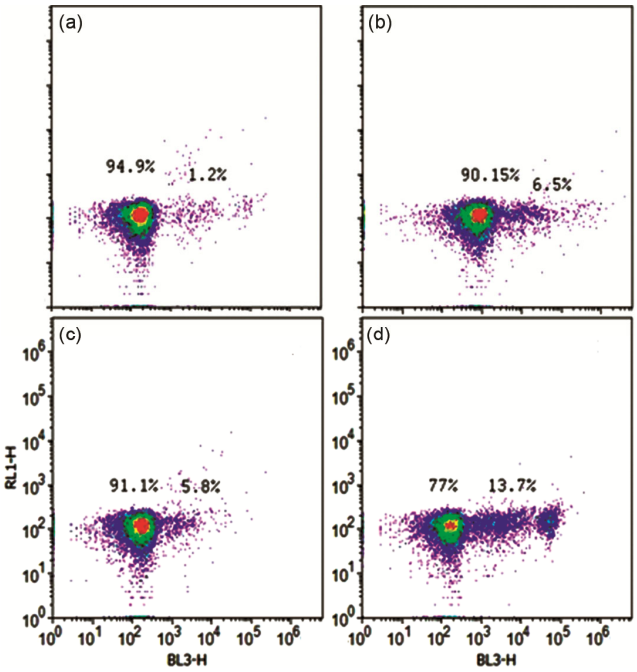


Fig. 3 — Poly caspases activation in NDI treated cells: (a) Untreated SKMEL2 cells, (b) **NDI-1** in SKMEL2 cells, **(c)NDI-2** in SKMEL2 cells and (d) **5-FU** in SKMEL2 cells. Flow cytometry of caspases induced by the NDI derivatives using the FAM Poly caspases staining method

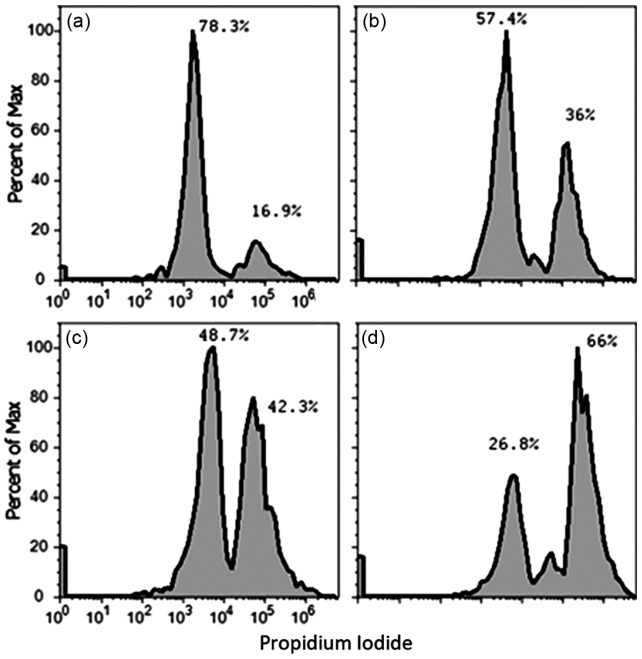


Fig. 4 — NDI treated SKMEL2 cells inhibition in G2/M phase (a) Untreated SKMEL2 cells (b) **NDI-1** in SKMEL2 cells (c) **NDI-2** in SKMEL2 cells (d) **5-FU** in SKMEL2 cells

apoptosis in the cells. The activated poly caspases activation leads to the irreversible apoptosis in the cells (Fig. 3).

Inhibition of cell cycle in cancerous cells

In normal cell growth and development, regulation of cell cycle progression plays key role. Many cell cytotoxic agents inhibit the cell cycle at the initial phases leading to cell death pathways. Deregulated expression of cyclin-dependent kinases activation is commonly observed in malignant cells. Cytotoxic agents activate, regulate cell cycle and cell cycle progression[16,17]. The effect of the NDI compounds on cell cycle progression in skin melanoma cells were investigated by propidium iodide assay. Cells were kept with 4 µM and 16 µM concentrations of NDI compounds for 24 h, analyzed for the population in cell cycle phase of subG1, G0/G1, S and G2/M (Fig. 4). NDI treated cells accumulated in G2/M phase with a decrease in G1 phase in the presence of NDI compounds in cells. 36% and 42.2% cells were found arrested in G2/M phase in **NDI-1** and **NDI-2** treated cells, respectively. The cell population from G1 phase was found decreased up to 57.4% in **NDI-1** treated cells (Fig. 4). The cell cycle inhibition was found at G2/M phase in **NDI-1** and **NDI-2** treated compounds. The Cdc2/cyclinB controls the G2/M transition and entry of cell into mitosis[17].

Flow cytometry of cell cycle: Expression of anti pro apoptotic phases

Mitochondrial membrane protein Bcl-2 is associated with the permeability membrane. Pro apoptotic proteins BH3- induces apoptosis by inhibiting Bcl-2 and Bcl-xL thus increasing membrane pores made by the death-promoting proteins- Bax and Bak1[18,19].

The regulation of the anti and pro- apoptotic proteins was studied by qPCR by using SYBR green chemistry. Anti-apoptotic Bcl-2 expression was found down regulated when **NDI-1** and **NDI-2** were present in SKMEL2 cells. Fig. 5 illustrates members of Bcl-2 family by regulation of the pro-apoptotic proteins. BAK1 expression was found to be more up-regulated in SKMEL2 cells when **NDI-1** and **NDI-2** were used. Both compounds down regulate Bcl-2, and increase the Bax genes in SKMEL2 cells, which was treated with **NDI-1** and **NDI-2** molecules for a day (Fig. 4a). Interestingly, Bax1 was increased when **NDI-1** and **NDI-2** were used as compared to the 5-FU. Expression of anti-apoptotic proteins was down regulated with **NDI-1** and **NDI-2** (Fig. 5), as compared with **5-FU**. Highly significant down regulation of Bcl2 and up regulation of Bak1 and Bax

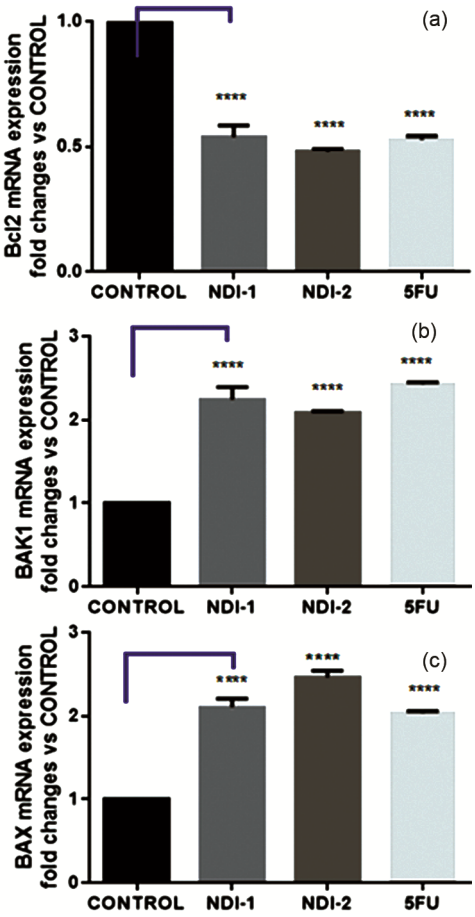


Fig. 5 — Anti- and pro-apoptotic genes expression in **NDI** treated cells. The antiapoptotic proteins Bcl2 and proapoptotic proteins Bak1 and Bax were analysed in presence of **NDI** in SKMEL2 cells. Error bars shows SD set as 100%; n = 3; ****P<0.0001, ***P<0.001, **P<0.01, *P< 0 .05 and NS-not significant.

was found in **NDI-1** and **NDI-2** treated cells. The Bax/Bcl-2 ratio also plays a significant role in apoptosis induction to promote cell death. Our results also suggest that up regulation of Bax and down modulation of Bcl-2 induces mitochondria mediated pathways through which both the derivatives (**NDI-1** and **NDI-2**) induce apoptosis. The Bax/Bcl-2 ratio initiate caspase signaling in apoptosis.

Discussion

In this study, we described new NDI derivatives (**NDI-1** and **NDI-2**), bearing phosphonate functionality, which inhibited SKMEL2 cell proliferation in a dose- and time-dependent manner. The unique structural combination of the NDI derivatives *i.e*. hydrophobic core, phosphonate head groups and an electron deficient nature, was found able to modulate chemo-resistance and to initiates apoptotic pathways in the SKMEL2 cells. In previous reports, several polyamine-modified NDI conjugates have shown more potent inhibition of cancer cells than amonafide both *in vitro* and *in vivo*[20]. The apoptosis in cancer cells was detected by cell viability assay. The live, apoptotic and necrotic cells were analyzed by fluorescence microscopy and flow cytometry. The expression of pro-apoptotic genes was found to be upregulated in SKMEL2 cells. NDI derivatives also down regulate Bcl-2 and upregulate the Bax proteins in SKMEL2 cells. The DNA strand breaks down in the cells, followed by expression of cMyc and Caspases, which confirms the irreversible apoptosis in SKMEL2 cells. In search of the apoptosis induction by **NDI-1** and **NDI-2**, a real time PCR assay was carried out. The expression and activation of the proapototic proteins is the activating step in the apoptosis[21]. NDIs treatment significantly triggered the expression of Bax proteins in SKMEL2 cells (Fig. 5). Bcl-2 plays an inhibitory role in mitochondrial apoptotic pathways[22]. As shown in Fig. 5, NDI treatment inhibited the expression of Bcl-2 protein. The breakdown of the mitochondrial membrane is the initiation of apoptosis induction by caspase[23-25]. The release of the pro-apoptotic proteins has a direct apoptotic effect on the mitochondria, and its activation induces cell cycle arrest and apoptosis through various pathways[25].

The results indicate that the composition/structure of the complex plays a significant role in modulating the cytotoxic effect on cells. For example, the population of apoptotic and dead cells was comparatively greater for **NDI-1** than **NDI-2**, thus the presence of a methylene group in the side chain bearing the phosphonate ester appears to improve the efficiency. Due to these structural differences, **NDI-1** and **NDI-2** were found to be more effective inducers of apoptosis in cancer cells than the control.

Further, detailed, mechanistic studies are required to define the effect(s) of NDIs on other human carcinoma cells, particularly on hormone-unresponsive cells, at molecular levels, and to assess the inhibitory effects in *in vivo* models of cancer. The present findings suggest that NDIs have great potential for development as therapeutic agents against skin cancer.

Conclusion

On the basis of the results, we have suggested a pathway by which NDIs (**NDI-1** and **NDI-2**) bearing

phosphonate functionality induce apoptotic cell death. The death of cells is induced by cell cycle inhibition and changes in Bax/Bcl-2 ratio for the mitochondrial apoptosis pathway. The Bax and Bak1 induces caspases dependent apoptosis. In this study, the NDI compounds induce cell death in human skin melanoma cells, with cell growth inhibition *via* G2/M-phase arrest and mitochondria mediated apoptosis. Further, detailed studies are needed to analyze the effect(s) of NDIs on other human carcinoma cells, particularly at the molecular level. The present findings suggest that NDIs have great potential for development as therapeutic agents against skin cancer.

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Authorship contribution statement

Kiran R. Kharat: PI, Conceptualization, Methodology, writing and corrections; Rajesh S. Bhosale: Methodology; Kamalakar P. Nandre: Methodology; Madan R. Biradar: Methodology; Sheshanath V. Bhosale: Synthesis data validation, conceptualization, writing and corrections; Sidhanath V. Bhosale: PI, conceptualization, writing and corrections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Information

Supplementary information is available in the website http://nopr.niscpr.res.in/handle/123456789/58776.

Compliance with Ethical Standards

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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