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ORIGINAL ARTICLE

Invitro Evaluation of Torin2 and 2, 6-Dihydroxyacetophenone in Colorectal Cancer Therapy

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Abstract Colorectal cancer (CRC) is one of the most prevalent cancers diagnosed worldwide. Despite recent advances, resistance to cytotoxic and targeted therapy remains one of the greatest challenges in long-term management of colorectal cancer therapy. Recently established role of mTOR signaling in proliferation of CRC has incited for evaluation of mTOR kinase specific inhibitors in CRC therapy. Second generation mTOR kinase inhibitors including Torin2 has demonstrated efficient anticancer properties against variety of cancers and are in various stages of drug development. The time and financial constraints concomitant from discovery to development of efficient chemical inhibitors has redirected attention towards investigation of wide spread naturally occurring largely inexpensive compounds for their therapeutic potential. One such naturally occurring compound acetophenone derivative polyphenolic compound 2, 6-Dihydroxyacetophenone (DHAP) inhibits cell growth in different conditions. We investigated anticancer properties of both Torin2 and DHAP against colorectal cancer in HCT8 cell lines. Both Torin2 and DHAP inhibited growth of CRC cells at different concentrations by restricting multiple cellular functions e.g., cell cycle progression, cell migration and induced apoptosis. Treatment of HCT8 cells with natural compound DHAP resulted in reduced expression of mTOR pathway specific genes p70S6K1 and AKT1. *In silico* docking studies showed affinity of DHAP to mTOR kinase like Torin2. Taken together, our result vouches for role of Torin2 in CRC therapy and recommends DHAP an mTOR inhibitor, as a potential lead in the development of new therapeutic regimes against colorectal cancer.

Keywords mTOR · Kinase inhibitor · Drug development · Docking · Natural Compound

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Introduction

Colorectal cancer (CRC) remains a significant cause of morbidity and mortality worldwide with high disease incidence. In spite of large-scale screening efforts recommended, significant number of patients are identified with advanced, metastatic disease [1, 2]. mTOR is a favourable drug target due to its inevitable role in cell growth and survival under diverse environmental conditions [3–5]. It not only executes the signals received from upstream PI3k/Akt pathway but also entertain responses from other signaling pathways. mTOR is a highly conserved serine/theonine kinase member of the phosphatidylinositol-3 kinase–like kinase (PIKK) family, which also includes ATR, ATM, DNA-PK, and SMG-1 [6, 7]. mTOR belongs to two complexes mTORC1 and mTORC2, largely regarded as rapamycin sensitive and insensitive complexes respectively. Loss-of-function mutations in tumor suppressors, such as PTEN, tuberous sclerosis ½ (TSC1/2), neurofibromin 1/2 (NF1/2), or oncogenic mutations in KRAS, PIK3CA, or AKT leads to hyperactivation of mTOR signalling in various cancers [8]. Far described and tested mutations such as S2215Y from a colorectal sample and R2505P from a kidney

sample, 33 other novel mTOR pathway-activating mutations established mTOR as an oncogene [9, 10].

Thorough investigation over the last few years has revealed the suppression of CRC upon inhibition of mTOR via rapamycin, siRNA and ATP kinase inhibitors [11, 12]. Rapalogs caused growth arrest in APC deficient CRC, implicating therapeutic benefit for high risk patients [13].The efficacy of the dual kinase inhibitors NVP-BEZ235 and PP242, in CRC cell line-derived xenografts have also been demonstrated [14]. The partial success with mTOR kinase inhibitors has been owed to intrinsic resistance of a large proportion of CRC cell lines and hence recommends for screening of more inhibitors [15, 16].Recently, Torin1, mTOR kinase inhibitor, has shown promising effects on growth of Colon cancer stem (CSCs) like cells. It has been proposed for clinical trials based on its growth inhibitory properties and induced apoptosis in CSCs cells. Analog of Torin1, Torin 2 is a novel, PIKKs inhibitor with a superior pharmacokinetic profile, potency and selectivity for mTOR [17]. It displays remarkable antiproliferative activity across a panel of cancer cell line (hepatocellular carcinoma (HCC) cell line, human B-pre ALL cell line, papillary thyroid cancer cell line, epithelial ovarian cancer cell line). It exhibits cytotoxic activity with an IC50 in the nanomolar range, induced G0/G1 phase cell cycle arrest, causes both apoptosis and autophagy by affecting mTOR pathway [18–21].

The cost and time associated with the development of potent mTOR specific inhibitors argues to search for known natural compounds with growth inhibitory properties [22, 23]. Polyphenolic compounds are a complex group of compounds that have attracted attention of late because of their widespread occurrence on plants and their significant biological activities [24].Previous reports have demonstrated the potential of polyphenolic compounds in inhibition of hepatic stellate cells proliferation [25].The role of phenolic hydroxyl groups on the anti-tumor promoting activity using benzoic acid derivatives has also been observed. 2, 6-Dihydroxyacetophenone (DHAP), acetophenone derivative of polyphenolic compound harbours antitumor promoting effects on the *in vitro* activation of EBV-EA. Also it lowers the differentiation of 3 T3-L1 preadipocytes and triglyceride accumulation in maturing adipocytes, and nitric oxide production in RAW 264.7 cells [26, 27]. Here we investigated the efficacy of DHAP in suppression of growth of CRC by inhibition of mTOR signalling. Initially in vitro anticancer properties of DHAP and Torin2 were studied in HCT8 cells (Human colon cancer cell line) that are known to harbour inherent resistance to apoptosis induced by the specific Akt inhibitor triciribin, 5-fluorouracil [28].The mechanistic details of growth inhibition by suppression of mTOR pathway were acquired by expression analysis of mTOR regulated genes and *in silico* studies.

Material and Methods

In vitro Cellular Assays Methodology

Reagents and Cell Culture Conditions

DHAP and Torin2 were procured from Biochem and Sellek Chemicals. Cell culture media, fetal bovine serum (FBS), trypsin, and 1% penicillin and streptomycin were purchased from Sigma Ald-rich or HiMedia. Cultures were maintained at 37 °C with 5% CO2 in a humidified incubator. HCT8 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin (100 units/ml), streptomycin (100 mg/ml). Cells were routinely detached with 0.1% trypsin and the cell number was counted on a haemocytometer before seeding for experiments. Cells were exposed to different concentrations of DHAP and Torin2. Cells incubated in culture medium with an equivalent amount of vehicle DMSO (final DMSO concentration was <0.2%) served as controls.

Quantitative RT-PCR (qRT-PCR) Analysis

Two lakh cells were seeded into the 24 well plate having culture media supplemented with 10% FBS and 1% penicillin/streptomycin and incubated at 37 °C. Cells were exposed to 80 μM, 100 μM DHAP and 1 μM Torin2 for 48 h. Total RNA was isolated by using MACHEREY-NAGEL RNA isolation kit. RNA quantified at 260/280 nm with thermo scientific nanodrop 2000 spectrophotometer. The absorption ratio A260 nm/A280 nm between 1.90 and 2 was taken into consideration for cDNA preparation. First strand cDNA was synthesized from 1 μg of total RNA with reverse transcriptase (Biorad) according to manufacturer instructions. Quantitative real time PCR was carried with SYBR green PCR master mix in Bio-Rad CFX96™ real time PCR machine (Bio-Rad Laboratories Inc., New Delhi, India). Dissociation curve was generated at the end of each PCR to verify that a single DNA species was amplified. The following cycling parameters were used: start at 95 °C for 5 min, denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and a final 5 min extra extension at the end of the reaction to ensure that all amplicons were completely extended and repeated for 40 amplification cycles. The following gene specific primers for HPRT Fwd: TATGGCGA CCCGCAGCCCT, Rev.: CATCTCGAGCAAGACGTTCA G, BCL-2 Fwd:CATGTGTGTGGAGAGCGTCAA, Rev.: GCCGGTTCAGGTACTCAGTCA, BID Fwd: GCTGTATA GCTGCTTCCAGTGTA, Rev.: GCTATCTTCCAGCC TGTCTTCTC, MMP9 Fwd: TTGACAGCGACAAG AAGTGG, Rev.: GCCATTCACGTCGTCCTTAT, AKT1 Fwd: CTGCAGCTATGCGCAATGTG, Rev.: TGGCCAGC ATACCATAGTG, and P70S6K Fwd: CTGGAAGC CTTGGAATGGG, Rev.: GCATCTATTTAAAAAAATC

were used for RT PCR analysis. HPRT was amplified as the reference gene to calculate fold change for genes of interest and to assure equal loading of the sample. All quantitative PCR experiments were performed in triplicate. Graph pad prism 5.0 was used for further analysis of real time PCR data.

MTT Assay

The cytotoxic effect of each drug was assessed by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. 3×10^3cells were seeded into a 96 well plate and allowed to adhere overnight. After 24 h incubation, media was removed and replaced with fresh medium containing different concentrations of compounds. The working concentration of DMSO did not exceed 0.2%. After treatment for 48 h, the medium was removed and washed with PBS and 20 μl of the MTT (0.5 mg/ml) was added to each well of the plate. Plates were incubated at 37 °C for 4 h. After incubation 200 μL of DMSO was added for solubilization of formazan and mixed very well and left in the dark for 10 min. The intensity of the color developed was recorded at 555 nm in fluorescence microplate reader (Synergy /H1).The inhibition rate (%) was calculated as (1): inhibition (%) = (1-$A_{555,compound}$ / $A_{555,control}$). Graph pad prism 5.0 was used for further analysis of MTT assay data.

Wound Healing (Gap-Closure) Assay

Two lakh cells were seeded in a 24 well plate and grown overnight for adherence. Next day, to distinguish cell migration from proliferation mitomycin C (0·01 mg/ml) (Sigma-Aldrich) was added to inhibit proliferation. After 6 h of mitomycin C treatment, cells were treated with different concentration of compounds prepared in fresh medium. Confluent cell monolayers were scratched with a 200 μl pipette tip to create a wound. The cells were then carefully washed with culture medium to remove free floating cells. DHAP and Torin2 were added at different concentrations and incubated for 48 h. To analyze rate of wound closure randomly chosen fields were photographed at 10× magnification with an inverted microscope, and the images were taken at identical locations at the 0 h and 48 h (adding scale bars to images). Percent cell migration was calculated by comparing final gap area to initial gap area using ImageJ.

Cell Cycle Analysis

Cells (2×10^4/well) seeded in 24 well plates were incubated with IC50 concentrations of each inhibitor for 24 h. Cells were washed with 1× PBS and trypsin was used to detach the cells. Subsequently suspensions were transferred to microtube and washed twice with phosphate-buffered saline (PBS) at 3000 rpm for 5 min. Cells were resuspended in PBS and then supplemented with cold absolute ethanol to final concentration of 70% followed by overnight incubation at −20 °C. Further ethanol was removed by centrifugation at 4500 rpm for 10 min. Cells were washed with 500 μl PBS. Finally in the darkness, cells were stained with 0.5 ml of warm PI solution, (7 ml of PI solution consist of 0.35 ml of PI stock (1 mg/ml), 0.7 ml of RNase (1 mg/ml), 6 ml of PBS), for 30 min. Samples were analyzed in BDbiosciences FACs machine.

Nuclear Morphology Analysis

Cell nuclear morphology was evaluated by fluorescence microscopy following DAPI staining. Cells (2×10^4/well) were treated with inhibitors for 48 h. The cells were washed with PBS (pH 7.4), fixed with 4% paraformaldehyde/PBS for 15 min at room temperature and finally samples were stained for 5 min with 1 μg/mL DAPI. The cells were then washed with PBS. Stained nuclei were photographed under a fluorescence microscope with a 40× objective (Olympus).

In silico Characterization Methodology

The compound was modelled using BUILD application of Maestro. The geometry was optimized by molecular mechanics using IMPACT in a dynamic environment using standard TIP4P water model. The energy minimization was done using Optimized Potentials for Liquid Simulations 2005 (OPLS 2005) force field with Polak-Ribier conjugate gradient and Truncated Newton conjugate gradient algorithms. The convergence threshold used was RMS gradient of 0.01. Conformational models of the ligand were generated.

Docking of the ligand with the available crystal structure 4JSV was carried out using extra precision (XP) method called GLIDE (Grid-based Ligand Docking with Energetics). The receptor grid generation for docking was done using both the Centroid of selected active site residues of kinase domain as well as blind docking. The results of both the methods were similar. The different conformations of the ligands were docked flexibly. The analysis of the poses, complexes and the binding affinities between the receptor and ligands were analysed using Schodinger's suite. The binding affinities and non-bonded interactions of DHAP were compared with that of Torin2.

Drug Likeliness Prediction

In silico prediction of ADME properties was done using QikProp. The compound DHAP was evaluated based on the atom types and charges, molecule's volume, surface area and rotor counts along with the physical descriptors such as lipophilicity, solubility and fraction absorbed in humans. Molecular weight, polar surface area, calculated log P, number of rotatable bonds, number of hydrogen-bond donors and

acceptors, log HERG (HERG K+ channel blockage), Log S (aqueous solubility) Log K_p (predicted skin permeability), Molecular volume (total solvent accessible volume), percent human oral absorption; log BB (predicted brain/blood partition coefficient) and the solvent accessible volume were the properties that were investigated. Lipinski rule of five was used for the drug likeness of the lead molecules.

Statistical Analysis

Analysis of Variance (ANOVA) and t-test were used to analyze all the data. GraphPad Prism *v.*4.0 (GraphPad Software Inc., San Diego, CA) was used for all statistical analysis [29]. Tuckey test was used to statistically analyze the mean values of the experiments. The significant difference was considered when probability (p value) ≤0.05. Data are presented as the mean ± SD from at least three independent experiments.

Results

DHAP and Torin2 Inhibits Cell Proliferation of CRC *Invitro*

Torin2 with favourable pharmacokinetic properties than its analogue Torin1 has yet not been studied in CRC therapy. Cytotoxicity of Torin2 and DHAP on HCT8 cell lines was estimated by MTT assays. Survival rates of the HCT8 cells are shown in Fig. 1a, b following exposure to Torin2 and DHAP respectively. A significant difference in growth from the control group was found at all tested concentrations at 48 h. Torin2 and DHAP showed concentration-dependent anti-proliferative effects. Though, the growth inhibitory effects of DHAP were observed at a concentration of 5 μM and above, survival rates declined gradually between 5 and 40 μM with a steep decrease at high concentrations (20 and 40 μM). Torin2 was more effective at lower concentrations i.e. 0.1, 1, 5, 10 μM, apparently mean 50% growth inhibition occurs at 0.1 μM of Torin2 and 20 μM of DHAP after 48 h of incubation.

Largely, altered expression of cell cycle regulators and the subsequent deregulation of the cell cycle plays crucial role in tumour promotion and are the most consistently found events in human malignancies including CRC [30]. TOR inhibitors are known to curtail cancer cell growth by arresting cell cycle progression e.g., Rapamycin functions by arresting cells in G1 phase. Torin2 arrests cells in G0/G1in B-pre ALL cell line, in papillary thyroid, epithelial ovarian cancer cells. In case of Hela cells, Torin2 induces a dose-dependent decrease in G1 cells and an increase in S-phase, sub-G1 phase cells, and cell death [17, 20].Thus Torin2 and DHAP were also tested for their effect on cell cycle progression of HCT8 cell line. Cells were treated with 1 μM concentration of Torin2 and 40 μM of DHAP for 24 h for cell cycle analysis. Interestingly, both Torin2 and DHAP arrested HCT8 cells in G0/G1 phase of cell cycle with 15% and 10% of reduction in percentage of cells in the G2/M phase respectively (Fig. 1c, d).

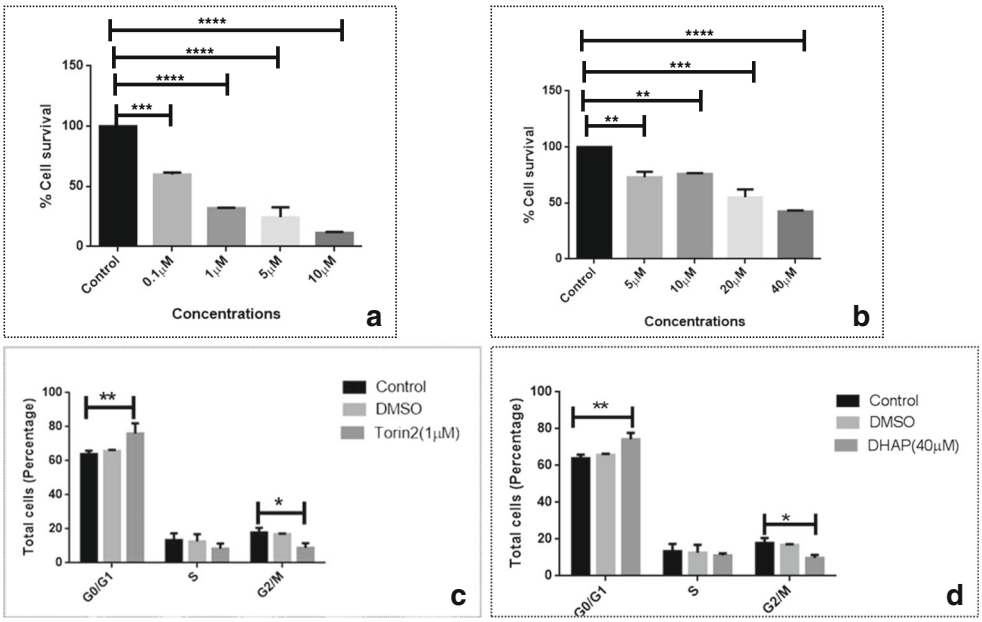

Fig. 1 DHAP and Torin2 inhibit cell growth and proliferation of HCT8 *invitro*. (**a**) Treatment with different concentration of Torin2 (0.1 μM, 1 μM, 5 μM, 10 μM), and (**b**) DHAP (5 μM, 10 μM, 20 μM, 40 μM) inhibits cell growth in the HCT8 colorectal cells in a dose dependent fashion. Cells were incubated for up to 48 h and then analyzed by MTT assay. Affect of (**c**) Torin2 (1 μM) and (**d**) DHAP (40 μM) treatment inhibits cell cycle progression in colorectal cells. Quantitative measurement of the distribution of cell cycle in HCT8 cells was done. Representative plots of thee independent experiments were performed. *$P < 0.05$ **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ versus controls

DHAP and Torin2 Induces Cell Death Via Apoptosis

Inhibition of mTOR induces apoptosis in colon cancer cells [17]. Further Torin2 is known to induce apoptosis at higher concentration via mTOR inhibition [17]. Thereby to analyse if DHAP exposure also promotes apoptosis, mRNA expression of pro-apoptotic gene (BID) and anti-apoptotic gene (BCL2) expression was predisposed by RTPCR. After 48 h of DHAP treatment the BCL2 expression was reduced and BID expression was induced (Fig. 2a-b). Furthermore, support to induced apoptosis came with analysis of nuclear morphology, using DAPI staining. It revealed the morphological changes associated with apoptosis, such as chromatin condensation and nuclear fragmentation in treated cells. This observation allowed for clear discrimination of treated cells from untreated non-apoptotic HCT8 cells, which have a normal, round, and unpunctuated nucleus (Fig. 2c). The percentage of apoptotic HCT8 cells was 17% at the concentration of 1 μM of Torin2. The percentages of apoptotic HCT8 cells were 6%, 9% and 15% at the concentrations of 40, 80 and 100 μM, respectively of DHAP (Fig. 2d).

DHAP and Torin2 Suppresses Colorectal Cancer Cell Migration

In addition to inducing uncontrolled growth of CRC cells activated mTOR pathway plays crucial role in invasiveness characteristics of a tumour such as cell proliferation, migration, and survival [31]. Thus drugs with antimigratory properties are preferred for anticancer therapy [32]. To test the inhibitory effect of DHAP in cell migration we carried out wound healing (gap closure) assays in HCT8 cells. A scratch/wound was drawn at the centre of the well before exposure of cells to different concentrations of drug DHAP (10, 20, 40 μM) or Torin2 (1, 5, 10 μM). After 48 h, untreated HCT8 cells migrated to the scratched area and closed the wound by forming colonies during the 'healing' process (Fig. 3a (i-ii), b (i-ii). However, following 48 h in contrast to untreated control cells,

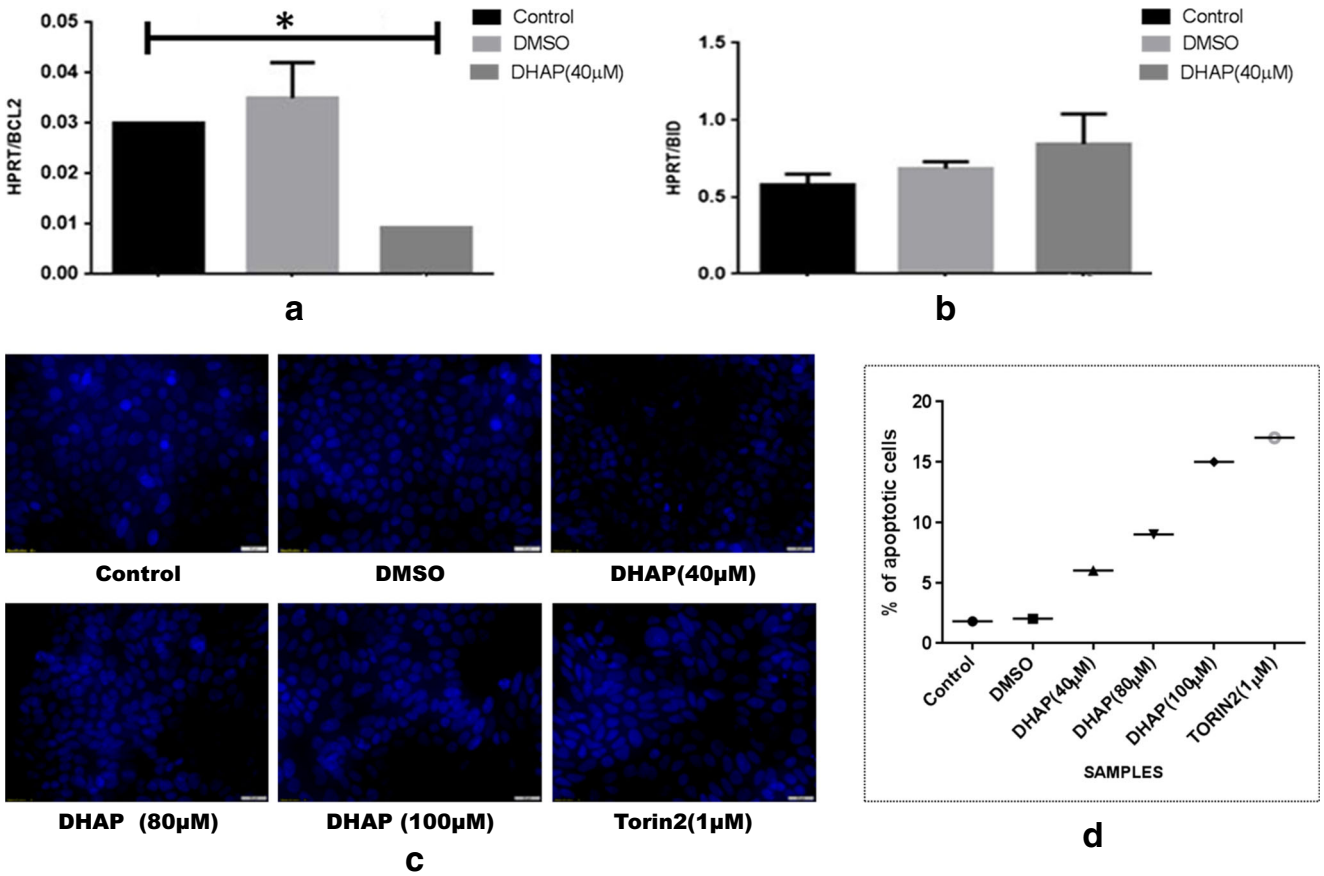

Fig. 2 DHAP and Torin2 induce cell death via apoptosis. mRNA levels of (**a**) BCL2 and **(b)** BID were determined by RT-PCR and normalized to HPRT as the loading control Cells were treated with DHAP (40 μM),incubated for up to 48 h.. DHAP down-regulated the expression of anti-apoptotic gene Bcl-2 but up-regulated the expression of pro-apoptotic gene BID.(**c**)Changes in cellular nuclear morphology was analyzed by using fluorescence microscopy. Cells were treated for upto 48 h with 1 μM concentration of Torin2 and 40 μM, 80 μM, 100 μM concentration of DHAP. DHAP and Torin2 treatment affects nuclear morphology in colorectal cells. Control cells were uniformly strained blue with unpunctuated nucleus, while Torin2 and DHAP-treated cells had condensed and fragmented nuclei. (**d**)Quantitative analysis of apoptotic cells upon treatments. Representative plots of thee independent experiments were performed. *$P < 0.05$ versus control

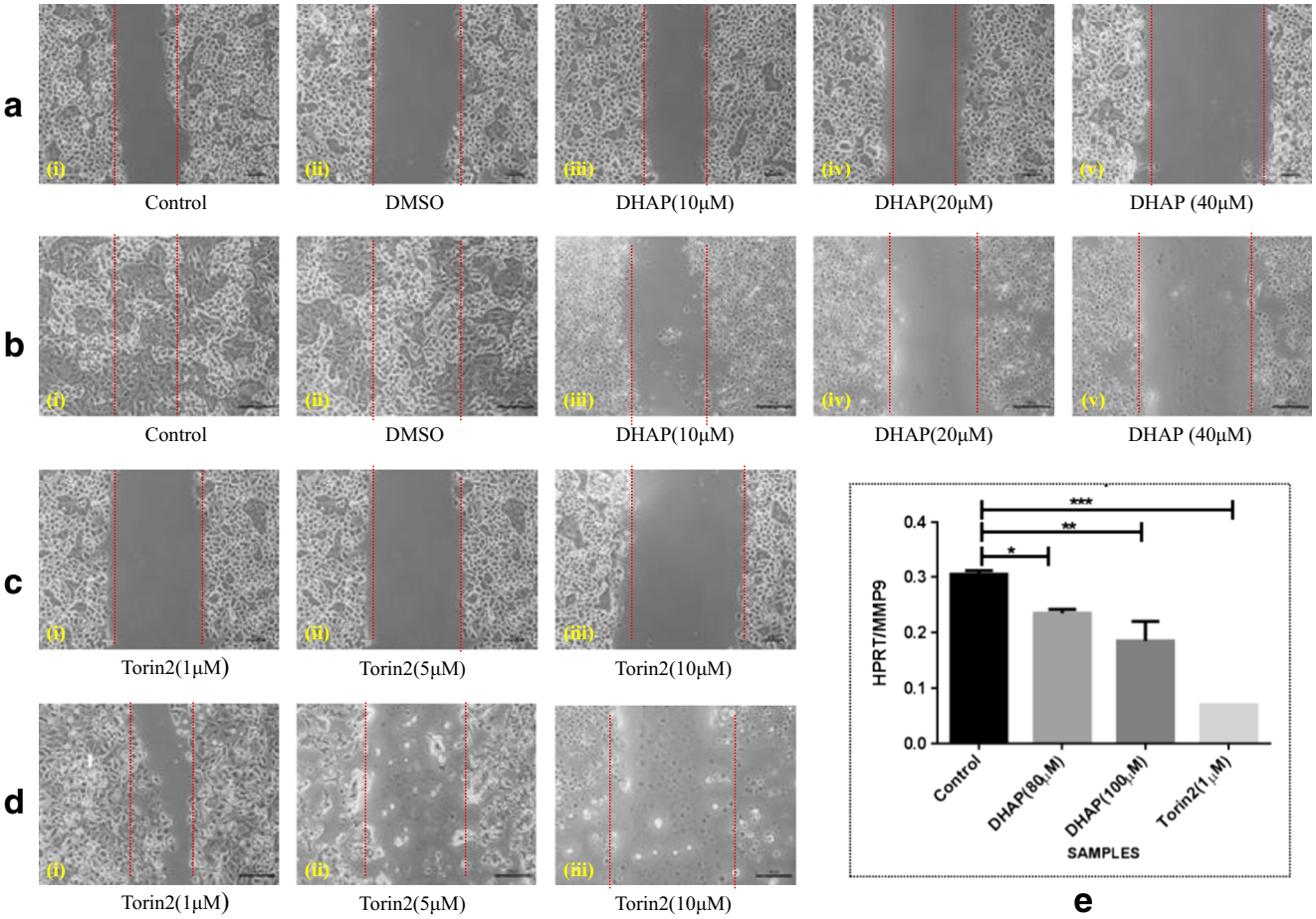

Fig. 3 DHAP and Torin2 suppress colorectal cancer cell migration. Cell migration or wound closure in the HCT8 cells was analyzed by wound-healing assay. Confluent HCT8 cells were wounded/scratched and incubated for 48 h after treatment with different concentrations of DHAP(**a,b**) and Torin2(**c,d**) along with control untreated cells. Images were captured at 0 h (**a,c**) and 48 h(**b,d**). The rate of wound closure was analyzed adding scale bars to images using ImageJ. (**e**) DHAP and Torin2 reduce the expression of MMP9 in colorectal cells. Cells were treated with 80 μM, 100 μM concentration of DHAP and 1 μM Torin2 for up to 48 h. MMP9 mRNA levels were determined by RT-PCR and normalized to HPRT as the loading control. Representative plots of thee independent experiments were performed. $*P < 0.05$, $**P < 0.01$and $***P < 0.001$ versus controls

the wound area persisted in the DHAP and Torin2 treated cells (Fig. 3a (iii-v), b (iii-v), c(i-iii), d(i-iii). Higher the wound area lesser is the cell movement. Thus both Torin2 and DHAP suppress metastasis of HCT8 cells. Further validation on cell migration inhibition was gained by assessing the expression of invasion-linked matrix metalloproteinase-9 (MMP9) in treated cells. In agreement, the expression level of MMP9 was relatively reduced in the treated HCT8 cancer cells compared to untreated control group. DHAP treatment suppressed MMP9 in a dose-dependent manner with upto 2 fold decreased MMP-9 expression at a concentration of 100 μM. Similar reduced expression of MMP9 in HCT8 cell lines was observed upon treatment with Torin2 (Fig. 3e).Apoptosis is a basic physiologic process in wound healing [33]. Remarkably, apoptotic morphological changes e.g. cells loss of contact with their neighbours, rounded shape and detachment from the surface pronounced membrane blebbing were also observed in CRC cell line at different concentrations of Torin2 and DHAP.

DHAP Prevents Tumorigenesis and its Progression through Binding to mTOR Kinase Catalytic Site

Essentially, the success of kinase inhibitor is outcome of suppression of activity of both mTORC1 and mTOC2 complexes. These complexes have been shown to be over expressed in CRC. It's established that in a carcinogenic state, mTORC1 activation leads to subsequent activation of P70S6K1, activated p70S6K1 inhibits mTORC2. mTORC2 functions upstream of Akt and activates Akt by phosphorylation of Akt at Ser473 [34, 35]. In support, the role of down-regulation of mTORC2 on reducing proliferation of colon cancer cell lines and inhibition of the formation of tumor xenografts *in vivo* has been deciphered earlier [36]. Previously expression profiles of AKT1 and p70S6K1 have been demonstrated as molecular marker for role of mTOR based pathogenesis and progression in panel of Gastric cancer cell lines such as AGS, MKN28 and MKN45 [37, 38]. Gene expression analysis of cells treated

with Torin2 and DHAP showed reduced expression of AKT1, P70S6K1 relative to the corresponding untreated control HCT8 cells (Fig. 4a-b). Thus like Torin2, DHAP inhibits CRC growth by inhibiting mTOR signalling.

Further insights into mTOR specific inhibition by DHAP were gained by *in silico* studies. The mTOR crystal structure available in PDB (4JSV) was used for analysing the binding of DHAP to mTOR as compared to Torin2. The available crystal structure had following domains: Kinase domain from 2115 to 2431, FAT domain 1381-1981, mTOR 1376-2549, FRB domain 2021-2118, FATC domain 2432–2549 [39].Computational docking was used to sample the conformations of DHAP at protein binding sites using GLIDE [40, 41]. To assess which of these conformations best complements the protein binding sites, scoring function was used. Docking accuracy which recognizes the true binding mode of the Ligand to the target protein was used to assess the quality of the docking methods. The Glide score was selected as the scoring function and the E-model (Kcal/mol) was used to rank the poses of the ligands as the E-model combines the Glide score, nonbonded interaction energy and excess internal energy of generated ligand conformation for flexible docking. The docking score of DHAP (−6.68) was comparable to that of Torin2 (−5.04). Both DHAP and Torin2 bound to the same catalytic site of mTOR. The major interacting residues in mTOR and Torin2 complex were Trp 2239, Ile 2356, Ile 2237, Met2345 and Leu2185 (Fig. 4c). These interactions were also prominent in mTOR and DHAP complex. A strong hydrogen bond was formed between backbone nitrogen of Val2240 and oxygen of DHAP (Fig. 4d). Figure 4e depicts DHAP bound in the active site of mTOR.

Predicted ADME Properties

The compound DHAP followed Lipinski rule of five. The drug-likeness rules defined in Qikprop were satisfied. The average molecular weight was 152.15 g/mol. The average number of hydrogen bond donors and acceptors were 2 and 3 respectively. The predicted octanol/water partition coefficient (QPlogPo/w) was 1.1 which is in the acceptable range

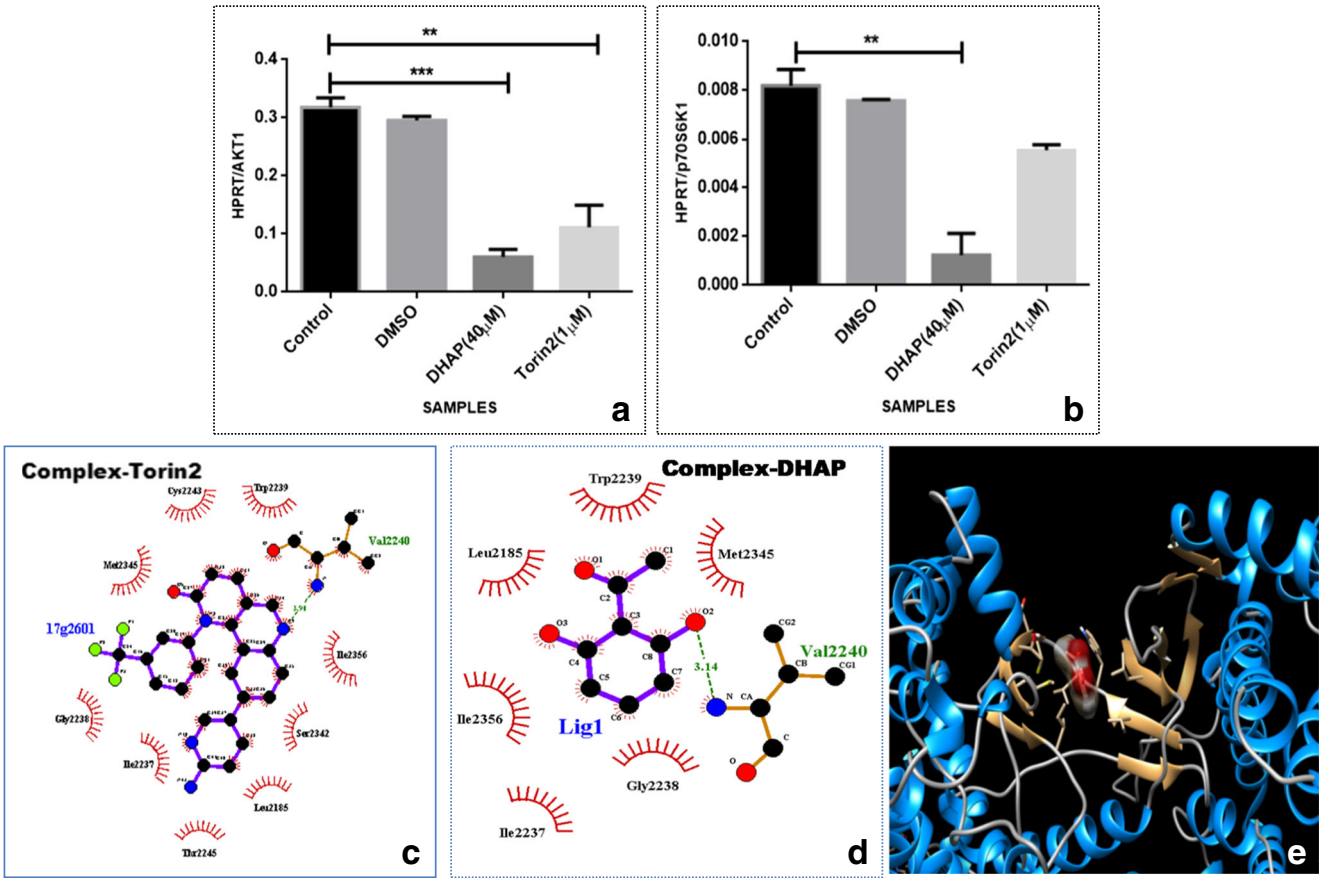

Fig. 4 DHAP prevents tumorigenesis and its progression through binding to mTORkinase catalytic site. mRNA levels of (**a**)p70S6K1 and (**b**) AKT1 were determined by RT-PCR and normalized to HPRT as the loading control Cells were treated with DHAP (40 μM) and Torin2(1 μM) incubated for up to 48 h.. DHAP down-regulated the expression of p70S6K1 and AKT1 upon inhibition of mTOR complexes. (**c**) Interaction of Torin2 in complex with mTOR (**d**) Interactions of DHAP in complex with mTOR using Ligplot. (**e**) DHAP bound to the active site of mTOR. DHAP is shown as spacefill model. mTOR is represented as ribbons and its interacting residues are shown in wireframe

i.e., −2.0 to 6.5. The average predicted aqueous solubility was 1.41e + 00 mg/ml having LogS value of −2.03. High gastrointestinal absorption was predicted. Also, the compound did not have problematic substructures suggested from Pan Assay Interference Compounds (PAINS).

Discussion

In spite of large-scale screening efforts recommended for early diagnosis and treatment, colorectal cancer (CRC) endures as one of the most widespread and deadly tumor types worldwide. The activity of PI3k/Akt pathway in CRC has earlier been targeted by different mTOR inhibitors including rapamycin and PI3ks kinase dual inhibitors. Torin1 has been proposed for clinical trials based on its growth inhibitory properties and induced apoptosis in CSCs cells. The efficacy of tested available inhibitors is often challenged by various intrinsic mutations that accumulate with progression of CRC and hence drives the need for development of drugs effective against resistant CRC.

Torin2 with better pharmacokinetic properties inhibits the progression of colorectal cancer cell lines. Interestingly like Torin1, Torin2 inhibits growth and proliferation of CRC cell line HCT8 by arresting cells at G0/G1 phase at nanomolar concentration. It inhibits migration and invasion of CRC cells by inhibiting movement of cells and results in cell death by inducing apoptosis. This implicates that Torin2 inhibits the progression of CRC cells and hence should be tested in clinics for CRC therapy.

Previously the role of natural plant products on inhibition of progression of CRC has been shown. Curcumin from plant *Curcuma longa* alters CRC growth by modulating Akt/Mtor signaling [42]. Another natural compound, pomegranate polyphenolics suppress azoxymethane-induced colorectal aberrant crypt foci and inflammation by suppressing the miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR pathways [43, 44]. Here we observed that natural compound DHAP also inhibits CRC growth. Like Torin2, DHAP showed strong growth inhibitory properties on HCT8 cell lines. DHAP inhibits cell proliferation by arresting cell cycle progression at G0/G1 phase and induces apoptosis at higher concentrations. Further DHAP also has role in control of metastasis as in wound healing assay no any movement of cells was observed in wounded HCT8 cells. Additionally a reduced expression of invasion marker gene MMP-9 suggests that exposure to DHAP reduces cells invasion ability. Thus DHAP not only arrest cells in G0/G1 phase but also curtails the migratory and proliferative abilities of CRC cells and kills cancerous cells by inducing apoptosis over a period of time. Further the reduced expression of p70S6K1 and AKT1 in DHAP treated CRC cells indicated the DHAP inhibits progression of CRC by inhibiting both mTORC1 and mTORC2 complexes like TORIN2. *In silico* studies demonstrated high binding affinity of DHAP to mTOR catalytic subunit. Molecular Docking showed DHAP interacts with Trp 2239, Ile 2356, Ile 2237, Met2345 and Leu2185 residues of mTOR, which are also shown to interact with Torin2. Interestingly earlier interaction studies of Torin2 with mTOR has predicted that interaction with Trp2239 is responsible for its high specificity towards mTOR kinase compared to other PI3KKs and for its efficacy at nano molar concentration [17]. The similar interaction of DHAP with this residue suggests high specificity of DHAP towards mTOR kinase compared to other members of PI3K family. The specific molecular docking, favourable ADME properties and inhibition of cellular growth process by DHAP recommend DHAP as potential mTOR inhibitor. Further studies will illustrate the mechanism of inhibition of mTOR signalling by DHAP. The DHAP can be used alone or in combination with other inhibitors for treatment of CRC patients with acquired resistance to available drugs. We anticipate its antitumor activity against other mTOR mediated cancers as well. Natural compound DHAP is a suitable candidate to be used as scaffold for development of more potent derivative with better pharmacokinetic properties and activity at nanomolar scale.

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Compliance with Ethical Standards

Conflict of Interest Authors declare no conflict of interest.

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

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