Synergistic effect of metformin and vemurufenib (PLX4032) as a molecular targeted therapy in anaplastic thyroid cancer: an in vitro study

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Abstract

Background Survival rate of patients affected with anaplastic thyroid carcinoma (ATC) is less than 5% with current treatment. In ATC, BRAFV600E mutation is the major mutation that results in the transformation of normal cells into an undifferentiated cancer cells via aberrant molecular signaling mechanisms. Although vemurafenib is a selective oral drug for the BRAFV600E mutant kinase with a response rate of nearly 50% in metastatic melanoma, our study has showed resistance to this drug in ATC. Hence the rationale of the study is to explore combinational therapeutic effect to improve the efficacy of vemurafenib along with metformin. Metformin, a diabetic drug is an AMPK activator and has recently proved to be involved in preventing or treating several types of cancer.

Methods and results Using iGEMDock software, a protein–ligand interaction was successful between Metformin and TSHR (receptor present in the thyroid follicular cells). Our study demonstrates that combination of vemurafenib with metformin has synergistic anti-cancer effects which was evaluated through MTT assay (cytotoxicity), colony formation assay (antiproliferation evaluation) and suppressed the progression of ATC cells growth by inducing significant apoptosis, proven by Annexin V-FITC assay (Early Apoptosis Detection). Downregulation of ERK signaling, upregulation of AMPK pathway and precision in epithelial-mesenchymal transition (EMT) pathway which were assessed by RT-PCR and Western blot provide the evidence that the combination of drugs involved in the precision of altered molecular signaling Further our results suggest that Metformin act as a demethylating agent in anaplastic thyroid cancer cells by inducing the expression of NIS and TSHR. Our study for the first time explored cAMP signaling in ATC wherein cAMP signaling is downregulated due to decrease in intracellular cAMP level upon metformin treatment.

Conclusion To conclude, our findings demonstrate novel therapeutic targets and treatment strategies for undifferentiated ATC.

Keywords Anaplastic thyroid cancer · Metformin · Vemurafenib · Thyroid stimulating hormone receptor · Cyclic AMP signaling

Introduction

Thyroid cancer, a fifth most common type of cancer detected in women is rapidly increasing worldwide of which more than 50% of patients diagnosed with thyroid cancer are below 50 years. Women are more prone to develop thyroid malignancies than men with 3:1 ratio [1]. Well-differentiated tumors in thyroid gland are classified as papillary and follicular thyroid carcinomas whereas undifferentiated tumors are classified as anaplastic thyroid carcinoma. Majority of differentiated thyroid tumors respond well to therapy which includes surgery, chemotherapy and radiation. However treatment to anaplastic thyroid cancer is highly complex...
due to high malignant potential where patients succumb within six months of diagnosis despite intensive treatment approaches [2, 3]. Though previous studies developed molecular targeting treatment strategies for ATC like epidermal growth factor receptor [EGFR] [4], peroxisome proliferator activated receptor γ (PPAR-γ) [5], mTOR inhibitor [6], treatment efficiency for ATC still remains challenging due to poor response rate. Hence identifying a novel therapeutic approach and studying the underlying molecular mechanisms of undifferentiated thyroid cancer is highly desirable to improve the patient’s survival. The proto-oncogene BRAF which encodes a serine/threonine kinase, transduces regulatory signals via growth factors, hormones, cytokines and mediates ERK signaling cascade which play a major role in the regulation of cell proliferation, differentiation and mediates ERK signaling cascade which play a major role in the regulation of cell proliferation, differentiation and apoptosis whereas BRAFV600E mutation which is present in approximately 40% of the ATC and in several human cancers including, melanoma, thyroid and colon carcinoma results in aberrant/constitutive activation of ERK signaling which phosphorylates several nuclear transcription factors and induce tumorigenesis in several human cancers including, melanoma, thyroid and colon carcinoma [7–10].

Undifferentiated ATC is associated with poor expression of genes such as sodium/iodide symporter (NIS) gene, TSHR (Thyroid stimulating hormone receptor), TPO (Thyroperoxidase), TG (Thyroglobulin), FOXE1 and TTF1 [11] that are involved in iodine transport and organization. Loss of NIS and TSHR expression are due to methylation in their promoter region and hence tumors in ATC can no longer respond to radioactive iodine therapy or thyroid stimulating hormone (TSH) suppressive therapy [12]. EMT pathway which play an important role in embryogenesis and development is associated with invasion of cancer to distant organs, metastatic dissemination, and poor clinical prognosis in various human tumors including thyroid carcinomas [13, 14]. In ATC, deregulation of BRAF/MEK/ERK and AMPK pathways lead to upregulation of numerous oncoproteins and EMT progression which converts epithelial cell into a mesenchymal cell with higher motility capacity and are characterised by decreased E-cadherin and increased vimentin expression. [15, 16].

Vemurafenib (PLX4032) inhibits BRAFV600E serine-threonine kinase by selectively binding to its ATP-binding site and improve survival among patients with BRAFV600E mutant cancers [17–19]. Although vemurafenib plays a pivotal role in BRAF mutant driven tumorigenesis, not all tumor types respond uniformly to BRAF-targeted therapy for eg ATC, colon cancer. Hence the research demands to explore combinational therapy as it is successful in most of the cancer types [20]. Metformin; a potentiator of AMPK activity has been proven to inhibit tumorigenesis in different types of cancer. In epidemiological studies, it has been reported that diabetic patients taking metformin drug for the treatment are reported to have smaller thyroid volumes, reduced incidences of nodular goiter, and higher remission rates from thyroid cancer [21]. Nonetheless the exact mechanism by which metformin activates AMPK, and if AMPK is the primary therapeutic target of metformin, are still under argument [22]. Similarly role of cAMP/TSHR signaling which are involving in thyrocytes growth, function and iodide metabolism is not yet studied in ATC tumorigenesis [23]. Thus our aim is to study the signaling pathway-related targets which may provide exceptional opportunities for the development of novel treatment strategies for ATC. In our study, we have evaluated the anticancer efficacy of combinational drug treatment (vemurafenib and metformin) on ATC by delineating the molecular mechanism that are involved in the inhibition of cell proliferation and in the precision of iodide metabolising genes and EMT markers in anaplastic thyroid cells.

Materials and methods

Reagents

Metformin, 5’-Azacytidine (Aza), Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and β-Actin (ACTB) antibody were purchased from Sigma Aldrich. Vemurafenib (PLX4032) was purchased from Sigma Aldrich. Antibodies for ERK, p-ERK, AMPK, p-AMPK, NIS, E-cadherin and Vimentin were purchased from Cell signaling Technology, USA. Versene, D-luciferin and coelentrazine were purchased from Invitrogen. Annexin V-FITC kit Early Apoptosis Detection Kit was purchased from Cell Signalling Technology. LANCE cAMP kit was purchased from PerkinElmer, USA. pGL4.29: A constitutive human CRE expression vector was a kind gift from Prof Karunagaran Devarajan.

Molecular docking: protein structure retrieval and preparation

The three dimensional structure of the Thyroid Stimulating Hormone Receptor (TSHR) was retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/). The proteins’ unique PDB ID: 3G04 was saved in the protein data bank format with a resolution of 2.55 Å. The structure was modified using the BIOVIA Discovery Studio. The water molecules and the bound ligands were visualised and removed from the structure prior to docking and was stabilised with the addition of Hydrogen and Polar molecules. The modified structure was saved in the PDB format.
Ligand structure retrieval and preparation

The chemical compounds to be docked against the TSHR were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The three-dimensional structure of the drug Metformin, with the molecular formula C4H11N5 and molecular weight 129.16 g/mol was downloaded with its unique PubChem ID: 4091 in the SDF format. The ligand was then viewed and modified using the BIOVIA Discovery Studio and the converted form was saved in the Sybyl/MOL2 format. The 2-D and 3-D conformer of the drug is shown as follows.

Similarly, the three-dimensional structure of the drug Vemurafenib and de-methylating agent 5-Azaacytidine were retrieved from the PubChem database. Vemurafenib with the molecular formula C23H18CIF2N3O3S and molecular weight 489.9 g/mol was downloaded in the SDF format using the compound unique ID 42,611,257. The ligand structure was viewed using the BIOVIA software and was saved in the Sybyl/MOL2 format compatible to the docking software. The structure of the de-methylating agent 5-Azaacytidine was retrieved from the PubChem database. The molecular formula of the drug is C8H12N4O5 and the molecular weight is 244.2 g/mol, the structure was downloaded in SDF format using the unique ID: 9444. The ligand structure was viewed and verified using the BIOVIA visualiser and was saved in the sybyl/MOL2 format compatible to the docking software used.
GEMDOCK

The receptor TSHR and the ligands Metformin, Vemurafenib and 5-Azacytidine were docked using the iGEMDOCK V2.1 software suite which is a complete standalone docking and virtual screening tool. The software is advantageous as it gives both the interactive surface to prepare the binding site of the protein and also serves as a visualisation software to view the docked poses. The software docks each of the ligand molecule with the receptor using the in-house tool GEMDOCK. iGEMDOCK generates protein-compound interaction profiles of Electrostatic (E), Hydrogen-Bonding (H) and Van der Waals interaction (V). iGEMDOCK groups the compounds for analysis based on the pharmacological interactions between the compounds and the protein structure. Finally, the iGEM software ranks the binding energy of the compounds by combining the interactions and energy based scoring of GEMDOCK. In the iGEMDOCK software the opening page allows the upload of the receptor and the ligand molecules and the parameters were set to the default range. On completion the interaction profile is generated which can be visualised using the in-built rasmol. And the interaction binding and the energy are analysed using the BIOVIA Visualiser studio, which allows to view the bond length and the amino acid interaction profile.

Cell culture

T-238, BCPAP (BRAF mutant) and HTH7 (wild type) cell lines were obtained as a kind gift by Dr Manoj Garg, NUS, Singapore, which were maintained in RPMI 1640 media (Roswell Park Memorial Institute 1640 Medium) supplemented with 10% FBS (Fetal bovine serum) at 37° C in 5% CO₂, and Penicillin (100 units/ml)/streptomycin (100 μg/ml) (Gibco, Grand Island, NY, USA).

Cell proliferation assay/cytotoxicity assay

The antiproliferative/cytotoxicity effect of metformin, vemurafenib and the combination of both were evaluated using MTT assay. This is a colorimetric quantitative assay which is measured by quantifying the formation of formazan crystals due to reduction of MTT tetrazolium by live cells. Briefly, 5 × 10³ cells were seeded into each well of a 96 well plate and allowed to attach overnight. Then, cells were treated with metformin or vemurafenib or the combination of both and incubated for 24 and 48 h. The untreated cells were used as negative control. After 48 h of treatment, MTT solution (0.5 mg/ml) was added to each well and incubated for about 3 h. Dark blue formazan crystals formed, implying the presence of viable cells on solubilization with DMSO. Non-viable cells do not form those crystals. The absorbance was measured by a microplate reader at 570 nm.
Colony formation assay

Colony formation assay is used to demonstrate the colony forming capacity of a single cell during treatment. T238 cells were grown in 6-well culture plates (1000 cells/well) and after 24 h, they were treated with different concentration of metformin, vemurafenib and the combination of both. Treated cells were incubated at 37 °C and 5% CO₂ for 10–14 days till the formation of optimal clones. After incubation, cells were fixed with methanol and stained with Crystal violet.

Annexin V-FITC assay

Annexin V-FITC assay is used to detect early apoptosis where the cells were stained by both PI and Annexin V-FITC. Expression of phosphatidylserine, which occurs through the binding of Annexin V-FITC to the cancer line cell surface, indicates apoptotic cells. Further staining with propidium iodide, a non-permeable DNA dye, indicates necrotic cells. After 48 h of treatment with metformin, vemurafenib or the combination of both, T238 cells were detached by trypsin, washed with PBS and then resuspended with Annexin V binding buffer. To each aliquot of 96 µl cell suspension, 1 µl of Annexin V-FITC conjugate and 12.5 µl of propidium Iodide (PI) solution were added and incubated for 10 min on ice in the dark. This was diluted to a final volume containing 250 µl/assay with ice-cold 1X Annexin V binding buffer and analyzed using a BD FACSURE flow cytometer with an in-built BD FACSuite™ Software.

RT-PCR

T238 cells were treated with Metformin, Vemrafenib or the combination of both. After 48 h of treatment, total RNA from treated T238 cells was extracted by TriZol reagent as per the manufacturer's protocol (Invitrogen, USA). To estimate the mRNA levels, reverse transcription was done using oligodT based conversion of mRNA to cDNA and were amplified using appropriate gene-specific PCR primers. Detection and quantitation of EMT markers and internal control β-Actin were performed using DyNAmo ColorFlash SYBR Green qPCR Kit reagent (#F416L, Thermo Scientific, USA). Relative expression levels of genes analyzed were calculated using 2 − ΔΔCT method by normalizing with internal control β-Actin.

Western blot

T238 cells were treated with Metformin, Vemrafenib or the combination of both. After 48 h of treatment, total cell lysates were prepared by incubating the treated and untreated cells on ice with RIPA lysis buffer (150mMNaCl, 1%NP40, 0.5% deoxycholate and 1% SDS) for 1 h. Protein concentration was then quantified by following Bradford's method. 30µg of protein lysates were resolved on 10% SDS-PAGE gel and later transferred to PVDF membrane (Immunoblot, Bio-Rad) at constant current of 220 mA for 1–1.5 h. The membrane was probed by primary antibody, washed thrice with TBS-T and TBS separately and again probed with secondary antibody conjugated with HRP. Bands were visualized by using the Enhanced Chemiluminescence kit (Bio-Rad), detected by using ChemiDoc and analysed by densitometry (Image Lab, Bio-Rad).

Measurement of cAMP signaling and intracellular cAMP concentration in anaplastic thyroid cancer cells

cAMP signaling apart from thyrocyte growth in thyroid gland, play an important role in the expression of differentiation markers such as NIS, TG and TPO that are responsible for iodide uptake [11]. cAMP is an important second messenger in mediating the cell signaling and hence it is vital to study the cAMP signaling and to measure the intracellular cAMP concentration. Luciferase Reporter assay and PerkinElmer Lance cAMP kit.

Luciferase reporter assay

T238 Cells were transfected with pGL 4.29 luciferase construct which possess CRE (cAMP response element) and luciferase gene. The medium was changed next day, and cells were treated with Forskolin or Metformin or both. After 48 h, cells were lysed using passive lysis buffer and dual luciferase assay was performed using luciferase assay buffer containing 25 mM glycyglycine, 15 mM phosphate buffer at pH 8.0, 4 mM EGTA, 2 mM ATP, 1 mM dithiothreitol, 15 mM magnesium sulfate and 75 µM luciferin.
and internal control assay buffer containing 1.1 M NaCl, 2.2 mM disodium EDTA, 0.72 M KH2PO4, 0.44 mg/mL bovine serum albumin (BSA), 1.3 mM sodium azide and 1.43 μM coelenterazine. The luciferase activity was measured in a luminescence plate reader (Molecular Devices Inc., USA), with renilla luciferase as the internal control.

**Measurement of intracellular cAMP assay**

Intracellular cAMP concentration was measured by using the Lance cAMP kit (PerkinElmer Corp., Waltham, MA). T238 cells were seeded in 24 well plates and next day the assay was performed by dissociating them with versene and by suspending in assay buffer (HBSS, 5 mM HEPES, 0.5 mM IBMX, and 0.1% BSA) at 2.0 × 10⁶ cells/ml. Cells were added in white OptiPlate-384 microplate from PerkinElmer and the protocol was continued as per manufacturer’s instructions. Samples treated with Forskolin, Metformin or both were measured in triplicates using a PerkinElmer multiplate reader.

**Statistical analysis**

Each experiment was repeated at least three times. Concentration-dependent curves and statistical analyses were done with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Analyses included mean ± sem, one- or two-way ANOVA, and Bonferroni post-test wherever appropriate (*, P < 0.05; **, P < 0.01; and ***, P < 0.001). The differences between test and control groups were analyzed using the Graph Prism Program. T-test was used to calculate the P-value of two groups compared. Values of P ≤ 0.05 were considered to be statistically significant. Relative density of western blot bans were quantified using ImageJ software.

**Results**

**Molecular docking of drugs with thyroid stimulating hormone receptor (TSHR)**

Methylation of TSHR in their promoter region leads to poor TSHR/CAMP signaling which in turn leads to low expression of thyroid-specific genes including sodium iodide symporter (NIS) is frequently lost in thyroid cancer. Therefore
in order to analyze the drug targets at the TSHR protein; drugs such as Metformin, Vemurafenib and 5-Azacytidine were docked with TSHR using iGEMDOCK software and docked scores of those molecules were represented in Table 1, with their binding energy, Vanderwaal energy, electrostatic and hydrogen bond profiles. binding energies of the protein–ligand (drug) interactions is crucial to describe how fit the drug binds to the target macromolecule. Our study showed that drugs (Ligands) such as Metformin (binding energy score = -65.1694), Vemurafenib (binding energy score = -113.236) and 5-Azacytidine (binding energy score = -114.015) significantly docked into the binding pockets of TSHR protein. The results show that all the three compounds Metformin, Vemurafenib and 5-Azacytidine bind efficiently to the TSHR and the interaction profile of the drugs along with bond length is shown in Fig. 1a, b, c. Further in order to analyze the relationship between metformin and vemurafenib with the TSHR protein, the docked poses were combined and it was observed that both the drugs bind to similar binding site of the protein with a structural overlap as shown in Fig. 1d

**Effect of vemurafenib on different thyroid cancer cells**

Effect of vemurafenib on different thyroid cancer cell lines such as HTH-7 (BRAFWT ATC cell line), BCPAP (BRAFV600Emutant, PTC cell line) and T238 (BRAFV600Emutant, ATC cell line) was examined by MTT assay. Thyroid cancer cells were treated with different concentrations of vemurafenib and measured the cytotoxicity after 24 h, 48 h, 72 h and 96 h of treatment. Vemurafenib as a single agent inhibited the cell growth in BRAF mutant BCPAP papillary thyroid cells after 48 h of treatment whereas BRAF mutant T238 anaplastic thyroid cells showed resistance to vemurafenib and BRAF wild type HTH7 cells were also not be affected efficiently by vemurafenib as shown in Fig. 2a,b

**Effect of metformin on different thyroid cancer cells**

HTH-7, BCPAP and T238 cells were treated with different concentrations of metformin and measured the cytotoxic activity after 24 h, 48 h, 72 h and 96 h of treatment. Metformin did not show any effect on HTH7 cells (BRAFWT ATC cell line) and BCPAP cells (BRAF BRAFV600Emutant, PTC cell line) as shown in Fig. 2c whereas in T238 cells, single agent metformin inhibited cell growth after 48 h of metformin treatment with an IC50 value (50% reduction in cell viability) of 5 mM and demonstrated a concentration-dependent cytotoxicity profile as shown in Fig. 2d. Metformin cytotoxicity is not further increased after 72 h and 96 h of treatment. T238 anaplastic cells were used in subsequent experiments as vemurafenib-resistant anaplastic thyroid cancer cell line to study its sensitivity towards the combinational therapy of vemurafenib and metformin.

**Combination of metformin and vemurafenib increases the cytotoxicity of ATC cells**

Efficacy of Metformin and Vemurafenib on ATC as a combinational therapy and to compare the same with the individual therapy is demonstrated by treating T238 cells (BRAF mutant anaplastic thyroid cancer cell line) with different concentration of metformin/vemurafenib individually and with the combination of both at different time intervals 24 h, 48 h, 72 h and 96 h. Combinational therapy showed increase in cytotoxicity at 48 h when compared to individual therapy. Control (without any treatment) did not contribute to cytotoxicity (Fig. 2e).

**Metformin, vemurafenib and their combination inhibits colony formation in anaplastic thyroid cancer cells**

In order to determine whether metformin or vemurafenib or their combination possess antiproliferation effect, a colony formation assay or clonogenic assay was performed. T238 cells were treated with 5 mM (IC50) of Metformin, 50 µM Vemurafenib and the combination of both drugs and incubated for 14 days at 37 °C and 5% CO2. Combinational treatment showed drastic decrease in the ability of single cell to grow into colonies when compared to individual drug treatment. Untreated T238 cells were used as a control (Fig. 2f, g).

**Combination of metformin and vemurafenib induces apoptosis in anaplastic thyroid cancer cells**

Apoptotic cell death of anaplastic cells were analysed in flow cytometry using Annexin V-FITC assay. A single agent vemurafenib induced 20.84% of apoptosis in the BRAFV600E mutant cell line T238, however no effect was seen with single agent metformin treatment. Interestingly, combinational treatment (50 µM vemurafenib and 5 mM metformin) of these cells resulted in 65% apoptosis. These results imply that combinational treatment significantly induced cell death (three times) when compared to monotherapy (Fig. 2h, i).

**Study of iodide metabolizing genes involved in thyroid metabolism with and without treatment of vemurafenib, metformin and 5-Azacytidine**

Iodide metabolizing genes such as NIS (Na/I symporter), TSHR (Thyroid stimulating hormone receptor), TPO
Fig. 2 Anticancer effect of Vemurafenib and Metformin in different thyroid cancer cell lines. Cytotoxic effect of vemurafenib in different thyroid cancer cell lines a. HTH7 (BRAF<sup>WT</sup> ATC cell line), b. BCPAP (BRAF<sup>V600E</sup>mutant, PTC cell line) and c. T238 (BRAF<sup>V600E</sup>mutant, ATC cell line). Cells were treated with or without different concentrations of Vemurafenib as a single agent, incubated for 48 h and the percentage of surviving cells with respect to controls were determined by MTT assay. d. Dose dependent effect of metformin on T238 cells. T238 cells were treated with different concentrations of Metformin and measured the cytotoxicity using MTT assay after 48 h. e. Combinational treatment of T238 cells with different concentrations of vemurafenib along with 5 mM of Metformin, incubated for 48 h and the percentage of surviving cells with respect to controls (untreated) were determined by MTT assay. f. Antiproliferative effect of drugs were determined by treating T238 cells with 5 mM metformin, 50 µM vemurafenib or their combination and incubated for 14 days till the formation of colonies. Cells were then fixed with methanol, stained with crystal violet and analyzed using IMJ Edge software with Colony parameters as follows Background (radius = 80), Gaussian Blur (sigma = 2), Maximum (radius = 2), Minimum (radius = 3), Remove outliers (radius = 12), Analyze particles (Size: 50-Infinity, Circularity: 0.3–1). Exclusion size: Colonies excluded due to filtration of particles with circularity less than 0.3. g. Number of colonies formed in metformin, vemurafenib or the combination of both treated group were compared to the number of colonies formed in untreated control group. h. Cells were stained with Annexin V and PI after 48 h of treatment (without control) or with 5 mM metformin, 50 µM vemurafenib or their combination. and were analyzed by flowcytometry. Percentages are shown in relation to the control. i. Percentage of early apoptotic cells formed in metformin, vemurafenib or the combination of both treated group were compared to the number of colonies formed in untreated control group. Each data point represents the mean of at least three independent experiments in triplicates. (*, P < 0.05; **, P < 0.01; and ***, P < 0.001) vs control

(Thyroxperoxidase), TG (Thyroglobulin), FOXE1 and TTF1 were analyzed by quantitative Real time PCR in T238 cells relative to normal thyroid cell line N-Thy. The results demonstrate that the mRNA levels of the genes were very low as shown in Fig. 3a. In order to induce the expression of the silenced genes, T238 cells were treated with metformin at the IC<sub>50</sub> value of 5 mM and after 48 h of treatment, mRNA level was measured and the result demonstrates that metformin treatment significantly increased the mRNA levels of NIS, TSHR, TG, TTF1. It has been reported that low level of gene expression is due to methylation of genes and hence metformin treatment was compared with that of demethylating agent 5-Azacytidine (10 µM), wherein interestingly metformin treated cells expressed higher mRNA levels than 5-Azacytidine and reveals that metformin may act as a demethylating agent (Fig. 3b). To further activate the gene expression levels, cells were treated with combination of 5 mM metformin and 50 µM vemurafenib, however neither vemurafenib as a single agent nor the combination with metformin increased the expression levels (Fig. 3b). Simultaneously, protein level of NIS was also measured by western blot and the result confirmed that metformin treatment on T238 cells enhanced the protein level of NIS as shown in Fig. 4c.

Combinational therapy regularizes the altered EMT markers expression better than demethylating agent 5-Azacytidine

To regulate the EMT pathway in BRAF mutant anaplastic thyroid cancer, T238 cells were treated with 5 mM metformin, 50 µM vemurafenib or combination of both and with 10 µM of 5-Azacytidine. After 48 h of treatment, RT-qPCR was performed to evaluate the mRNA of E-cadherin and Vimentin. Combinational treatment significantly upregulated E-cadherin and downregulated vimentin whereas average effect was seen with single agent metformin treatment. However, no effect was seen with 5-Azacytidine and vemurafenib treatment (Fig. 4a, b). Simultaneously, western blot was performed to analyse the protein expression level wherein E-cadherin level is increased and Vimentin level is decreased as shown in Fig. 4c. The results thus confirm combinalional treatment aid to regulate the altered EMT pathway in anaplastic thyroid cancer cells.

Study of drug treatment on molecular signaling mechanism of anaplastic T238 cells

RAF/ERK signaling and AMPK pathway were studied by analyzing changes in phospho-protein levels of ERK and AMPK pathways to identify the effects of the single agent or combinational treatment of 5 mM Metformin and 50 µM vemurafenib in T238 cell line. Single agent metformin had moderate effects on pERK whereas combinational treatment synergistically downregulated pERK protein levels. Metformin as a monotherapy drug upregulated the pAMPK protein level whereas single agent vemurafenib and combinational treatment did not show any effect on pAMPK protein levels as shown in Fig. 4c. Thus the results demonstrate that metformin targets the AMPK pathway by increasing the p-AMPK protein level whereas combinational treatment targets ERK signaling by decreasing the p-ERK protein level in BRAF<sup>V600E</sup> mutant ATC.

Metformin downregulates cAMP/PKA signaling by lowering the intracellular cAMP level

T238 cells were transfected with CRE reporter plasmid pGL 4.29 and treated with an increasing concentration of forskolin (adenylly cyclase activator). Luciferase activity was measured after 48 h (Fig. 5a) in which the result shows that the cAMP signaling is increased with increasing concentration of forskolin in T238 cells. We hypothesized to explore the effect of metformin on cAMP signaling since our work demonstrates that metformin can increase the p-AMPK level. Hence T238 cells were transfected with plasmid pGL 4.29, treated with 30 µM Forskolin, 5 mM metformin or both and measured the luciferase activity after 48 h. Interestingly, our
results showed decrease in luciferase activity when the cells were treated with 30 µM Forskolin and 5 mM metformin which demonstrates that metformin inhibits the cAMP mediated signaling as shown in Fig. 5b. Subsequently in order to determine whether the metformin lowers the cAMP signaling by decreasing the intracellular cAMP level or by inhibiting the PKA mediated cascade, intracellular cAMP concentration was measured. cAMP standard curve assay was performed in 96 well microplates (each well with 3000 cells) in a total volume of 24 µl by using different concentrations of cAMP and the lance signal (TR-FRET) was measured at 615 nm on PerkinElmer EnVision as shown in Fig. 5c. Simultaneously, forskolin dose-responsive curve was performed on T238 cells with different concentration of forskolin as shown in Fig. 5d. 0.5 mM IBMX (Phosphodiesterase inhibitor) was used in all the assays in order to prevent the reduction of LANCE signal produced by cAMP. An assay is performed to determine the interference of metformin on intracellular cAMP level by treating the cells with 5 mM metformin with different concentration of forskolin. The fluorescence intensities were then measured at 615 nm and found the lance signals were decreased as shown in Fig. 5e. Lance signal counts at 615 nm obtained in cAMP standard curves will allow the determination of the amount of cAMP produced in stimulated cells [24]. The resulting signals were directly proportional to the cAMP concentration of the cells and our results showed that intracellular cAMP levels were reduced in metformin treated cells (Table 2) and thus our study confirm that metformin downregulates cAMP signaling by decreasing the intracellular cAMP level.

**Discussion**

Current treatment for undifferentiated and aggressive anaplastic thyroid cancer remains ineffective and are associated with incurability and patient mortality [25]. Majority of ATC has BRAF mutation; resulting in the unusual activation of ERK signaling, downregulation of AMPK signaling and induce EMT which in turn result in invasion and metastasis of cancer [26, 27]. Therapeutics blocking this signaling pathway; for instance BRAFV600E inhibitor vemurafenib works effectively and reduce tumor volume in some cancers however it did not show any promising effect on ATC which has BRAFV600E mutation [28]. Thus the limited success of current chemotherapeutic regimens for ATC has led...
to the investigation of novel therapeutic approach and targets for the treatment. Our rationale of the study is to consider metformin (FDA approved drug used routinely for diabetes) as a potential drug to combine with vemurafenib for ATC therapy due to two major reasons. 1. In normal cells, metformin exerts anti-mitogenic effect by activation of the AMP-activated protein kinase (AMPK) and by inhibition of the MAPK pathway at the cellular level to regulate cell growth which is an important factor in insulin/IGF signaling [21]. 2. Recently, in cancer cells it has been demonstrated that metformin possesses anti-tumorigenic properties and proven to inhibit cell proliferation, invasion and migration in most of the cancer cells. It appears to affect multiple key processes related to cell growth, proliferation, and survival [28–31]. To proceed with our hypothesis, Metformin, Vemurafenib and 5-Azacytidine were docked with TSHR protein using iGEMDock software to determine the docking scores in which the protein–ligand interaction was successful in identifying that the drugs were docked with TSHR with common binding site sharing between metformin and vemurafenib. Hence in the present work, combinational treatment (AMPK activator Metformin and BRAF inhibitor Vemurafenib) was studied on Anaplastic BRAF mutant cells in which our results demonstrated that combination of metformin with vemurafenib can inhibit the cellular proliferation followed by induction of apoptosis in ATC. Our results demonstrate metformin is targeting specifically on BRAF mutant anaplastic cells and did not show any effect on HTH7 cells (BRAFwt ATC cell line) and BCPAP differentiated cells (BRAFV600Emutant, PTC cell line), whereas vemurafenib inhibited the proliferation of BCPAP cells and did not show any significant effect on BRAF wild type

Fig. 4 qPCR was performed to estimate EMT markers and MMP-9 mRNA expression levels in T238 cells that were treated with or without metformin and 5-AZ cytidine (a) and cells that were treated with or without control metformin and vemurafenib or the combination of both (b). Whole cell lysates were obtained after 48 h treatment of metformin, vemurafenib or their combination, and expressions of ERK, p-ERK, AMPK, p-AMPK, NIS, E-cadherin and Vimentin were checked by Western blotting. β-Actin was used as an internal control (c). Western blots are developed in ChemiDoc western blot apparatus from BioRad. Density of protein bands were quantified with respect to control (d). *P<0.05, **P<0.01, and ***P<0.001, versus control
Fig. 5 Determination of cAMP signaling and intracellular cAMP level in ATC cells. T238 cells were transfected with pGL4.29, treated with 30 µM Forskolin or 5 mM metformin or both and measured the luciferase activity after 48 h to identify the effect of metformin on cAMP signaling in ATC. A standard curve for cAMP was performed by using different concentrations of cAMP diluted from the cAMP standard stock and measured the fluorescence intensity (Lance signal) at 615 nm. Cells grown in 24 well plates were harvested with a non-enzymatic cell dissociation solution versene and 3000 cells per well in 96 wells opti-plate were seeded and treated without or with different concentration of Forskolin and 5 mM Metformin with different concentration of Forskolin. After 2 h of treatment, Lance signal was measured at 615 nm on PerkinElmer EnVision instrument. Table 2. The resulting signals produced after Forskolin or Metformin or both were correlated with cAMP standard curve lance signals as they are directly proportional to the concentration of cAMP determined in cAMP standard assay. (*, P < 0.05; **, P < 0.01; and ****, P < 0.001) vs control.
HTH7 cells. Differentiated thyroid cancer has better prognosis because the cells express sodium-iodide symporter (NIS) and can be targeted by radioactive $^{131}$I whereas undifferentiated thyroid carcinomas do not express sufficient NIS and hence radioactive $^{131}$I will not be taken by cancer cells; thereby leading to difficulty in treating anaplastic cancer cells [32]. Iodide-metabolizing genes like NIS, TSHR, TG, TPO, FOXE1 and TTF are found to be methylated in ATC and hence their expression levels are silenced [33].

In our study, we showed metformin act as a demethylating agent by activating the expression of NIS, TSHR, TG and TTF in BRAF mutant anaplastic cells which was assessed by RT-PCR and Western blot. Several studies demonstrated that activation of AMPK causes cell-cycle arrest and shuts down processes that consume energy through modulation of several signaling pathways including cAMP and mTOR pathways [25]. In addition, AMPK as a cellular energy sensor are involved in catabolic pathways to replenish cellular ATP stores and result in the surge of cellular AMP/ATP ratio by inhibiting ATP synthesis and thereby preventing the activation of the enzyme adenylyl cyclase to synthesise intracellular cAMP [17]. These findings are consistent with our study that metformin activates AMPK pathway on BRAF mutant cells, down-regulates cAMP signaling and lowers intracellular cAMP level. Our results for the first time showed advances in identifying the molecular target (cAMP) in ATC. We have further proceed our study on investigating the molecular target (cAMP) in ATC. We have further proceeded our study on investigating the EMT process in anaplastic thyroid cancer cells.

**Conclusions**

Overall, our findings demonstrate that vemurafenib along with metformin targets and regulates multiple signaling pathways—BRAF/ERK signaling, cAMP signaling, AMPK pathway and EMT process in anaplastic thyroid cancer cells and significantly induce apoptosis. In addition, metformin act as a demethylating agent by activating the suppressed iodine metabolising genes of anaplastic thyroid cancer cells. Metformin ability to overcome the resistance of vemurafenib implies that this diabetic drug can be used along with well-established anticancer therapies in future to treat aggressive cancers and thus our work represents novel combinational therapy and therapeutic targets for ATC.

**Acknowledgements** The authors deeply thank BRNS, DST-Women scientist, Government of India for providing financial support for this research work. We sincerely thank Dr. D. Karunagaran, Head, Department of Biotechnology, Bhupat & Jyoti Mehta School of Biosciences, IIT Madras for allowing the access to the laboratory.

**Author contributions** VR and LD designed the study. LD conducted the literature search of the study and performed the experiments. SR performed Bioinformatics work. The study was supervised by VR, DK, KA and KR. VR and LD analysed and validated the data and wrote the manuscript. All authors had read the manuscript and approved the publication of this manuscript.

**Data availability** All data generated or analysed during this study are included in this published article.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

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