The mTOR inhibitor, rapamycin, sensitises solid tumour cell lines to isoprenoid induced toxicity.

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ABSTRACT

Protein prenylation is a post-translational addition of a lipophilic farnesyl or geranylgeranyl moiety derived from the pyrophosphate substrates, intermediates of the mevalonate pathway. Prenylation of Ras-related small GTP-binding proteins and heterotrimeric G proteins constitute protein activation events associated with cellular proliferation. The monoterpenes limonene and perillyl alcohol inhibit protein isoprenylation resulting in cell cycle arrest and induction of apoptosis. Clinical trials of d-limonene and perillyl alcohol resulted in dose limiting toxicity. Reducing the dosage of isoprenoids, while maintaining the anti-proliferative effect is a challenge. Interestingly, the limiting factor of the mevalonate pathway, HMG CoA reductase, is controlled at translation level and hence sensitive to mTOR activity. The purpose of this study was to investigate the dose response using a combinatory treatment of isoprenoids and rapamycin (mTOR inhibitor), on various solid tumour cell line models. 3 solid tumour cell lines, namely PC3 (prostate) C32 (melanoma) and A549 (lung), were chosen on the basis of sensitivity to the mTOR inhibitor, rapamycin. Cytotoxicity assays using XTT were performed on the cell lines treated with Limonene, Perillyl alcohol and Ŭ-Pinene. In this study XTT assays were used to quantify viable cells after treatment with Isoprenoids and Rapamycin alone or in combination. Dosages at IC50s were used to measure apoptosis by Annexin V staining and flow cytometry. The C32 and PC3 cell lines were sensitive to rapamycin treatment, resulting in a decrease in cell viability by more than 20%, but retaining a constant growth curve thereafter. A549 was not sensitive to rapamycin at all concentrations tested. For all combinatory treatments, 50ng/ml rapamycin was selected. The viability of PC3 and C32 cell lines decreased significantly by the combinatory effect of 50ng/ml rapamycin and isoprenoids. Although cell death was enhanced after sensitisation with rapamycin, other mechanisms of loss of cellular viability, other than apoptosis, have a major role. Our results show a statistically significant reduction in IC50 of various isoprenoids, following pre-sensitization with 50ng/ml of rapamycin in the prostate cell line (PC3) and the melanoma cell line (C32). Hence this novel combination of drugs targeting two mechanisms that converge on a common target, provide a higher efficacy compared to using either drug on its own. This merits further investigation to characterise the mechanism/s of viability suppression in the solid tumour cellular models.

INTRODUCTION

Protein activation involves the post translational modifications, prenylation and phosphorylation. Protein prenylation is a post-translational addition of a lipophilic farnesyl or geranylgeranyl moiety at a Cys residue derived from the pyrophosphate substrates, intermediates of the mevalonate pathway [1]. Prenylation of Ras-related small GTP-binding proteins, heterotrimeric G proteins and nuclear lamins [2] constitute protein activation events associated with cellular proliferation. The monoterpenes limonene and perillyl alcohol inhibit protein isoprenylation as a result of suppressed 3-hydroxy-3-methylglutaryl (HMG) CoA reductase activity, supporting the role of monoterpenes as anti-proliferative agents [2, 3]. Inhibition of HMG CoA reductase, results in cell cycle arrest and induction of apoptosis [4, 5]. In addition, limonene and its derivative perillyl alcohol inhibit NF-κB signaling promoting apoptosis [6], resulting in suppressed proliferation and metastasis of gastric cancer [7], mammary and pancreatic tumors [8]. Another monoterpene, α-pinene is a potent inhibitor of NF-κB and exerts its function as an inhibitor of inflammatory responses [9].

The anti-proliferative capacity of perillyl alcohol was investigated in several phase I trials in patients with high grade malignancies [10] and solid tumours [11] and Phase II trials in patients with metastatic prostate cancer [12], advanced ovarian cancer [13], and metastatic colorectal cancer [14]. Dose limiting toxicity of d-limonene and perillyl alcohol in clinical trials includes nausea, vomiting, hypokalemia, anorexia and eructation [11, 15]. Reducing the dosage of isoprenoids while maintaining the anti-proliferative effect, is a challenge.

mTOR inhibition has been combined with other agents to enhance antitumour effect of other therapies [16-18]. To support the proposed combination of inhibitors, previous studies show that simultaneous inhibition of the Ras/Raf/MEK/ERK (MAPK) and the PTEN/PI3K/AKT/mTOR pathways results in upregulation of pro-apoptotic molecules such as members of the BCL-2 family of pro-apoptotic proteins, like Bim and Bad [19]. In addition, co-targeting the ras/MEK and the AKT/mTOR pathway in malignant melanoma provide a treatment option for acquired resistance during therapy [20].

Of interest, the translation of the HMG CoA reductase transcript is sensitive to the availability of the eukaryotic Initiation Factor 4E (eIF4E) [21]. Hence the phosphorylation of the eIF4E binding protein, 4EBP1 ensures release of eIF4E for translation initiation of structured transcripts that are otherwise not translated [22]. 4EBP1 is phosphorylated by the mammalian target of rapamycin, mTOR. Inhibition of mTOR using rapamycin results in sequestration of eIF4E into a 4EBP1 complex and a shift from cap-dependent to cap-independent translation initiation. Studies using the in vitro cellular models for prostate and melanoma, characterized the deregulation of Akt and ras pathways as being central to the progression of the disease [23]. Growth and progression of these tumours is associated with constitutive activation of mTOR [24]. This supports the rationale to inhibit both the Akt/mTOR and ras pathways with potential synergistic effect to attenuate cell growth and proliferation. In this study we investigated a panel of tumour cell lines

for sensitivity to low dose rapamycin and selected 2 sensitive cell lines, namely the prostate cancer cell line, PC3 and melanoma cell line, C32 to study the synergistic effect of isoprenoids. The rapamycin resistant cell line A549 (basal epithelial cells) was included in the study.

MATERIALS AND METHODS

Cell lines, Media and Reagents: 3 solid tumour cell lines were chosen from a panel of cell lines. PC3 (ATCC CRL-1435; prostate) and C32 (ATCC CRL-1585; melanoma) cultured in DMEM + 10% FBS. A549 (ATCC CCL-185; lung epithelial) cultured in RPMI + 10% FBS. All cell lines were incubated at 37°C in a 5% CO_2/95% air atmosphere. Pharmacological inhibitors used include rapamycin (Alexis Biochemicals, San Diego, CA), Limonene, Perillyl alcohol (Sigma-Aldrich Chemie GmbH, Germany), Ŭ-Pinene (Fluka, Sigma-Aldrich Chemie GmbH, Germany).

Cytotoxicity assays using XTT were performed on all cell lines. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by mitochondrial dehydrogenases in viable cells. Cells grown in 96 microtiter plates for 24, 48 and 72hrs and were incubated with XTT solution and activator solution (Cell Proliferation Kit, Biological Industries, Beit Haemek, Israel) for 2-24hrs before reading on microplate reader. In the study XTT assays were used to quantify viable cells after treatment with Isoprenoids and Rapamycin alone or in combination. Rapamycin stock solution (1mg/ml) was used to yield 7 concentrations (25, 50, 75, 100, 150, 200, 300ng/ml), while a stock solution (400εg/ml) of each isoprenoid was used to prepare a range of concentrations (0, 2, 10, 20, 40, 60, 80, 160 ppm). The combinatory experiment involved addition of 50ng/ml rapamycin to the range of each test isoprenoid.

Annexin V/Propidium iodide apoptotic assay was used to measure the decline of viable cells and the appearance of early apoptotic and late apoptotic/necrotic cells in one assay. Cells were left in 6-well plates in duplicates and incubated with test drugs. IC50s obtained from XTT results were used for treatment of each cell line. Every cell line encompassed (1) steady state (SS) cells and (2) 50ng/ml Rapamycin treatment, (3) each isoprenoid added alone and (4) in combination with 50ng/ml rapamycin. 1 x 10^5ï 1 x 10^6 cells were harvested and centrifuged. 2mL suspension was centrifuged and the pellet was resuspended in 500uL 1X binding buffer (Annexin V-FITC Apoptosis Kit Plus, Biovision, Mountain View, CA 94043 USA). 5εL of Annexin-V antibody and 5εL Propidium iodide were added and cells were incubated for 5-15 minutes in the dark and read by flow cytometry (Bryte-HS, Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS AND DISCUSSION

This study aims to establish a combinatory treatment of isoprenoids with rapamycin, to improve efficacy of isoprenoid chemotherapeutic effect at lower dosages. The sensitivity to rapamycin was determined using XTT viability assays. The cell lines PC3 and C32 show a marked reduction of cell proliferation following addition of 50ng/ml rapamycin. This reduced proliferation was maintained at higher dosages, but rapamycin did not induce a cytotoxic effect, hence we score this as sensitization of the cell lines that retain cell cycle progression. Of interest, the PC3 and C32 cell lines have mutations in the phosphatase PTEN. PTEN mutations increase activity of PI3K/Akt/mTOR pathway and this can explain the sensitivity to the mTOR inihibtor, rapamycin. In this study, rapamycin sensitive cell lines, resulted in a decrease in proliferation at a concentration of 50ng/ml and a decreased ED50 in combinatory treatment with all isoprenoids tested. In contrast, cytotoxicity assays of A549 did not show sensitivity towards rapamycin alone, and combination of the three isoprenoids with rapamycin did not decrease ED50, possibly because the cell line is PTEN wild-type.

PC3 and C32 are sensitive to low dose rapamycin

To assess the sensitivity of the cell lines to rapamycin, we exposed the cells to different doses of rapamycin and measured the loss of viability at 3 time points (24, 48 and 72 hours). Of interest, in both PC3 and C32, a dose of 50ng/ml rapamycin resulted in a suppression of cell viability by more than 20% at 48hours and retained a constant viability at higher doses of the drug (Figure 1 A-B). This sensitization of the cell lines with 50ng/ml rapamycin is associated with hypo-phosphorylation of 4E-binding protein (4EBP; Figure 1D), which is a direct target of the kinase, mTOR (mammalian target of rapamycin). The phosphorylation status of the cells treated with rapamycin was compared to the steady state (ss) condition for each cell line. The hypo-phosphorylation of 4EBP following rapamycin treatment of C32 and PC3, sequestrate the eukaryotic initiation factor 4E resulting in suppression of translation initiation efficiency. The rapamycin resistant cell line A549 (Figure 1C; basal epithelial cells) was included in the study. An effective dose, ED50 was reached only in PC3 at 300ng/ml rapamycin at 72hours.

Perillyl alcohol is the most potent isoprenoid, suppressing cell viability at low doses

Isoprenoids induced a loss of viability greater than 50% (effective dose, ED50) in the 3 cell lines tested in this study. Perillyl alcohol was the most potent isoprenoid with an effective dose ranging from 16 to 25 ppm at 48hours, while the effective dose of Ŭ-pinene ranges from 32 to 54 ppm and that of limonene from 34 to 56 ppm at 48 hours (Figure 2). Interestingly, the prostate cell line PC3 required high dose of isoprenoids at 24 hours and was as sensitive as the other cell lines at 48 and 72 hours. There was no significant differences in the effective doses between time point 48 and 72 hours for the 3 cell lines, hence for the combinatory experiments measurement at 48 hours was selected.

Perillyl Alcohol was the most potent isoprenoid on all the 3 cell lines, confirming reports stating that Perillyl alcohol is more potent than Limonene and is one of the most potent inhibitors of proliferation [31]. Cotargeting PI3K/mTOR and Ras pathways with a combination of PI3K/mTOR and RAS signaling pathway (MAPK) inhibitors suggest that inhibition of growth was related to upregulation of proapoptotic Bim and Bad [19, 32].

Pretreatment with Rapamycin and combinatory experiments

Rapamycin was added at a concentration of 50ng/ml to sensitise cells 24hrs prior to the addition of isoprenoids. Addition of rapamycin, 24hours before treatment with isoprenoids, proved to be more effective than adding the drugs together (data not shown). The combinatory treatment of 50ng/ml rapamycin with isoprenoids resulted in decreased ED50 of the cell lines C32 and PC3 (Table1; Figure 3). The combination was most effective on the PC3 cell line.

Interestingly, PC3 and C32 were sensitive to rapamycin after 48hrs and not 24hrs, an observation that is supported in previous studies [25]. This was observed in cytotoxicity assays and in combinatory experiments. Rapamycin inhibits mTORC1, while mTORC2 is not affected and maintains Akt/PKB survival signaling. We propose lack of sensitivity during the first 24hrs to be Akt-driven, forcing survival signals through mTORC2, however, prolonged rapamycin treatment (48hrs and 72hrs) causes rapamycin to bind to newly synthesized free mTOR and interfere with mTORC2 assembly (Figure 4), downregulating Akt/PKB survival signaling [26].

Apoptosis in A549 cell line

A549 cell line showed a marginal apoptosis induction when sensitization with 50ng/ml rapamycin, resulting in 11.3% apoptosis induction with Perillyl alcohol, compared to 5.2% when Perillyl Alcohol was added alone. Similar to Perillyl Alcohol, Limonene had 11.9% apoptosis induction in combination compared to 3.4% when added alone (Table 2). Interestingly, although the viability assays indicate a significant decrease in viability following combinatory treatment in the PC3 and C32 cell lines, the cells do not undergo apoptosis induction as measured using Annexin V/PI staining.

In our study, early apoptosis induction was observed when A549 was treated with isoprenoids in combination with 50ng/ml Rapamycin. Since XTT experiments showed that A549 was not sensitive to rapamycin at 24hrs and 48hrs, apoptosis induction is probably neither due to inhibition of mTORC1 (at 24hrs) nor mTORC2 (at 48hrs), and is largely a result of isoprenoid inhibition.

Although cytotoxicity assays revealed sensitivity of PC3 and C32 towards rapamycin, apoptosis was not detected. Antiapoptotic proteins like Bcl-2 and Bcl-xL were reported to be highly expressed in PC3 cell line [27], and could be possibly induced by rapamycin treatment [28], supporting the lack of apoptotic signals observed in annexin-V assays. For future studies,

protein analysis of antiapoptotic proteins Bcl-2 and Bcl-xL in rapamycin-treated cell lines, and cell cycle analysis measuring growth arrest at G1 by flow cytometry could have been significant in identifying possible loss of apoptotic signals in PC3 and C32. A possible mechanism explaining our viability results is the induction of autophagy. Inhibition of mTOR was implicated in autophagy induction followed by cell death [29, 30].

CONCLUSIONS

Our results show a statistically significant reduction in ED50 of various isoprenoids, following pre-sensitization with 50ng/ml of rapamycin in the prostate cell line (PC3) and the melanoma cell line (C32). Hence this novel combination of drugs targeting two mechanisms that converge on a common target, provide a higher efficacy compared to using either drug on its own. This merits further investigation to characterise the mechanism/s of viability suppression in the solid tumour cellular models. Although the mechanism of loss of viability in our study is not yet identified, we hypothesise that addition of rapamycin to PTEN defective cell lines, sensitise the cells for autophagy induction, following addition of the isoprenoids. Of interest the rapamycin sensitized cell lines, have a significantly lower ED50 upon the addition of the isoprenoids used in the study (Table 1). Further studies are required to understand the mechanism of cytotoxicity and a larger panel of cancer cell lines shall be assessed for rapamycin sensitisation.

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FIGURE LEGENDS

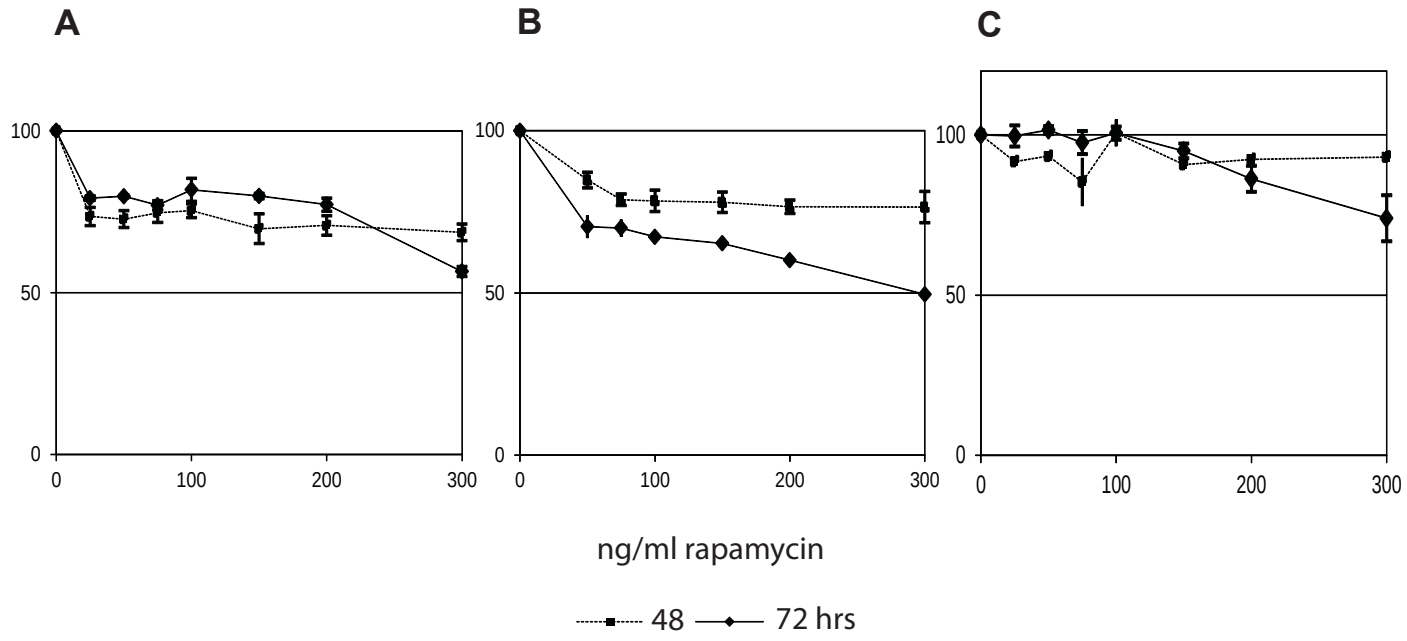
Figure 1. Sensitisation of PC3 and C32 using rapamycin. **A, B, C:** Cells were cultured in the presence or absence of rapamycin, using a concentration gradient from 0 to 300ng/ml. Each data point is an average of three independent experiments, with triplet measurement for each experiment. The standard error is indicated using error bars. The solid lines plot the percent viability after 72 hours following rapamycin addition, and the dotted lines after 48 hours. The melanoma **[C32; A]** and prostate **[PC3; B]** cell lines are sensitized by 50ng/ml of rapamycin. The basal epithelial cell line, A549 is resistant to rapamycin, even at high concentrations. **D**: Cells were treated with 50ng/ml rapamycin and harvested for total cell lysates. Western blots were stained with antibodies recognizing total 4EBP (4EBP Ab). The non-phosphorylated, hypo and hyper-phosphorylated proteins can be discriminated by their distinct electrophoretic mobility as α, β and γ-isoforms respectively. The steady state (ss) 4EBP phosphorylation status is also included for the 3 cell lines investigated [C=C32; P=PC3; A=A549].

Figure 2. Isoprenoid cytotoxic dosages. Cells were cultured in the presence of the isoprenoids perillyl alcohol, limonene and Ŭ-pinene, using a concentration gradient from 0 to 160ppm. Each data point is an average of three independent experiments, with triplet measurement for each experiment. The open squares plot the ED50 upon addition of Limonene; the open diamonds the ED50 upon addition of perillyl alcohol and the solid triangles the ED50 of Ŭ-pinene. The data show the time points 24, 48 and 72 hours after addition of the isoprenoid.

Figure 3. Cells were cultured in the presence of isoprenoids. The solid lines show viability of cells treated with isoprenoids only, while the dotted lines plot the viability of cells upon addition of isoprenoids following sensitisation with 50ng/ml rapamycin. Each data point is an average of three independent experiments, with triplet measurement for each experiment. The standard error is indicated using error bars. The basal epithelial cell line **[A549; A, D, G]**; the melanoma **[C32; B, E, H]** and prostate **[PC3; C, F I]** cell lines are treated with different isoprenoids, namely Perillyl alcohol **[A, B, C]**; Limonene **[D,E,F];** and with Ŭ-pinene **[G, H,I].** All data points are taken following 48 hours of isoprenoid addition.

Figure 4. Proposed effect of prolonged rapamycin treatment at 24hrs and 48hrs. Proliferation of cells continue for the first 24hrs of rapamycin treatment due to survival signals channeled through mTORC2/Akt pathway. Prolonged rapamycin treatment will inhibit mTORC2 assembly and consequently Akt/PKB survival pathway, inhibiting cell proliferation.

Figure 1


A
B
C
100
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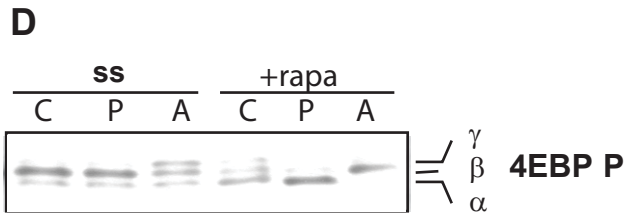
0
100
200
300
ng/ml rapamycin
48
72 hrs


D
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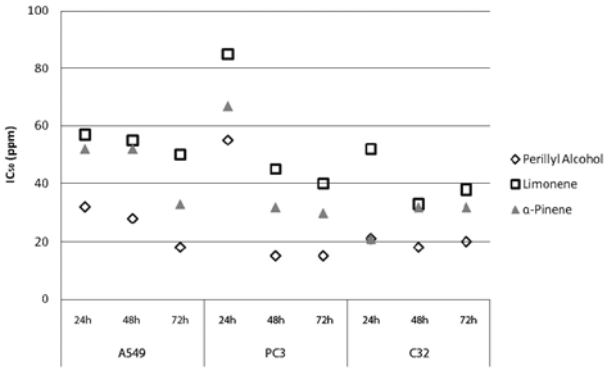
4EBP P

Figure 2


100
80
60
40
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IC50 (ppm)
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48h
72h
24h
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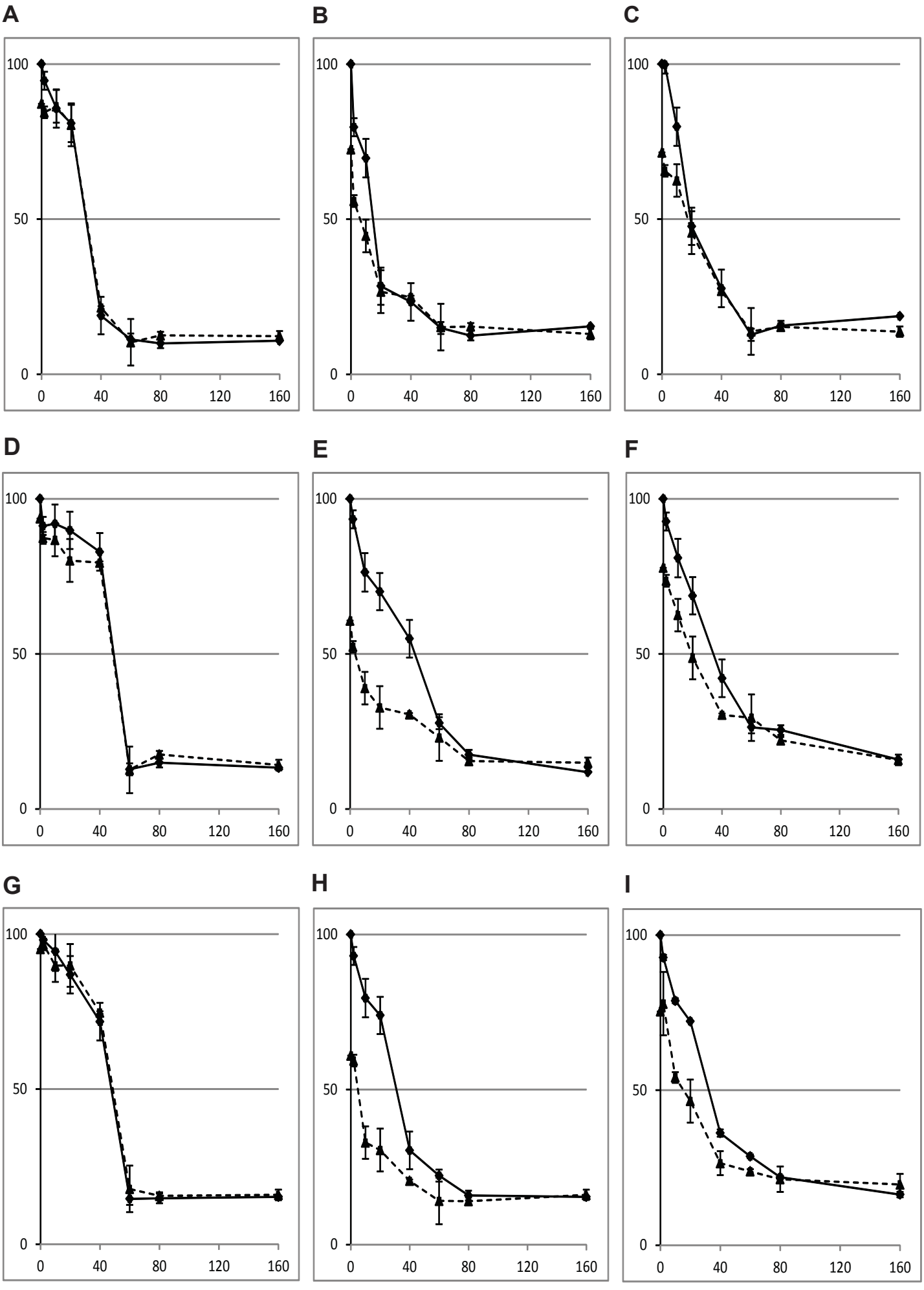
48h
72h
A549
PC3
C32
Perillyl Alcohol
Limonene
α-Pinene

Figure 3


A
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combination
isoprenoid alone

Figure 4

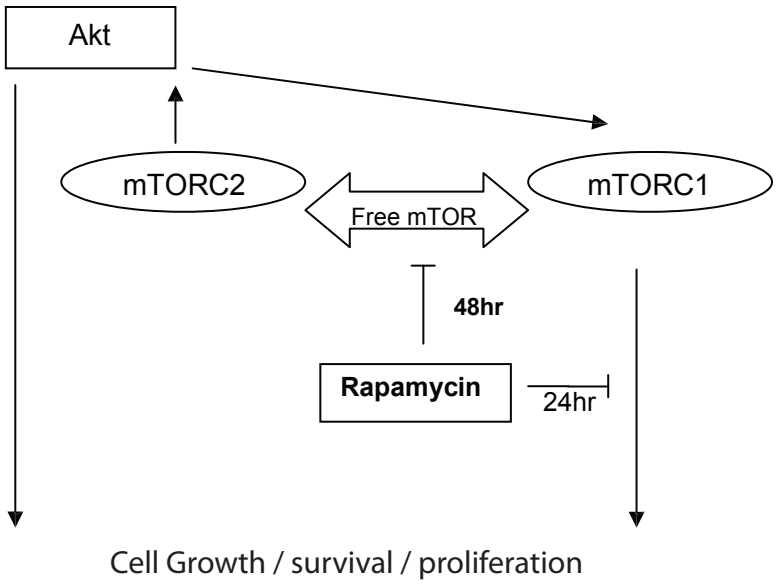
Akt
mTORC2
Free mTOR
mTORC1
48hr
Rapamycin
24hr
Cell Growth / survival / proliferation

Table 1

IC50 of cell lines treated with isoprenoids alone or in combination with rapamycin.

	isoprenoid	rapamycin	24h	48h	72h
A549	Perillyl Alcohol	-	32	28	18
		+	30	28	22
	Limonene	-	57	55	50
		+	54	55	52
	α-Pinene	-	52	52	33
		+	49	52	33
PC3	Perillyl Alcohol	-	55	15	15
		+	55	5	12
	Limonene	-	85	45	40
		+	67	5	5
	α-Pinene	-	67	32	30
		+	67	5	5
C32	Perillyl Alcohol	-	21	18	20
		+	18	18	20
	Limonene	-	52	33	38
		+	22	18	23
	α-Pinene	-	21	32	32
		+	8	15	8

Table 2. **Apoptosis induction in A549**.

Percentages indicate AnnexinV +ve and PI -ve (early apoptosis induction) cells. Conditions include cells treated with Perillyl Alcohol, Limonene and α-Pinene alone and in combination with rapamycin (rapa; 50ng/ml).

[Steady state (ss) means untreated]

Condition		Annexin V +ve (%)
Steady State (ss)		1.4
50ng/ml rapamycin		1.7
Perillyl Alcohol	Alone	5.2
	+rapa	11.3
Limonene	Alone	3.4
	+rapa	11.9
α-Pinene	Alone	1.9
	+rapa	2.1