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Resveratrol-induced apoptosis in human T-cell acute lymphoblastic leukaemia MOLT-4 cells

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ABSTRACT

Resveratrol (RES) is a natural occurring phytoalexin that has been shown to have chemopreventive activity. Resveratrol acts both by suppressing cell proliferation and inducing apoptosis in a variety of cancer cell lines. In this study, we show that RES induces apoptosis in MOLT-4 acute lymphoblastic leukaemia cells by modulating three different pathways that regulate cells survival and cell death. We show for the first time that RES inhibits the survival signalling pathways Notch and their down stream effector and modulates the operation of interacting signalling systems. It induces an increase in the levels of the pro-apoptotic proteins p53, its effector p21waf and Bax. We also show that RES inhibits the PI3K/Akt pathway and activates Gsk-3β. The data presented here demonstrate unequivocally that RES induces apoptosis by inhibiting the Notch pathway and markedly influencing the operation of the interacting apoptosis pathways mediated by p53 and PI3K/Akt. These data support findings from other laboratories that have suggested the use of RES as a chemopreventive agent. Here, we have identified potential signalling pathways influenced by RES and this could lead to the identification of the targets of RES-induced apoptosis and growth control.

1. Introduction

Current attempts to improve the survival of cancer patients largely depend on strategies to target tumour cell resistance, chemoresistance and late relapses, which are the hallmarks of acute lymphoblastic leukaemia (ALL). Notwithstanding, in recent decades conventional chemotherapy has produced a dramatic improvement in survival of patient with ALL [1]. However, refractory or relapsed disease continues to pose a serious problem. In particular, T-ALL is an aggressive form of cancer that affects children and adolescent and patients whose ALL cells exhibit in vitro resistance to anti-leukaemic agents, who have a substantially worse prognosis than patients whose ALL cells are drug sensitive [2–4]. It is now widely accepted that the apoptotic capacity of the cancer cells is crucial in determining the response to chemotherapeutic agents and that defects in the core machinery of the apoptotic pathway contribute to chemoresistance and poor outcome in patients with T-ALL [5].

In recent years, many compounds that occur in the diet and beverages have been identified as potential chemopreventive agents. Among them is the hydroxylated stilbene RES

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(3,4′,5-trihydoxystilbene), which belongs to a class of defense molecules called phytoalexins. These are produced by several plants in response to stress, injury, UV irradiation and fungal infections. RES is normally found in many dietary products such as grapes, peanuts, berries and wine [6]. Jang et al. [7] described the cancer chemopreventive activity of RES, which inhibits cellular events associated with tumour initiation, promotion and progression. These authors demonstrated that RES can inhibit free-radical formation and reduce oxidative and mutagenic stress. Recently many other studies have confirmed the ability of RES to suppress the proliferation of a variety of human cancer cell lines and to induce apoptosis in in vitro assays [8]. It has been shown that RES acts by modulating the activity of many different signalling pathways involved in cell cycle regulation and survival, including the PI3K (phosphoinositide-2 kinase)/Akt pathway.

Akt acts down stream to PI3K to regulate many biological processes, such as proliferation, apoptosis and growth. A relevant target for Akt is glycogen synthase kinase-3 (Gsk-3), which acts as a positive modulator of apoptosis. In unstimulated cells, constitutively active Gsk-3 phosphorylates different proteins, such as several transcription factors necessary for cell survival, promoting their inactivation or degradation. Akt is responsible for mediating the inhibition of Gsk-3β by catalysing the phosphorylation of the Ser9 residue in the amino terminus of Gsk-3β [9]. Recently, it has been shown that Gsk-3β can phosphorylate Notch and regulate its activity [10].

Notch is a transmembrane receptor that mediates intracellular signalling involved in cell differentiation and cell survival. Notch signalling is initiated through receptor–ligand interaction, which leads to the proteolytic cleavage of the intracellular domain of the receptor (NIC) and to its translocation to the nucleus. NIC binds to Cbl and modulates the transcription of genes involved in cell differentiation and cell survival. Notch signalling plays an important role in hematopoiesis and constitutively active intracellular forms of Notch have been shown to have oncogenic activity in T-ALL [11,12]. Activated Notch signalling confers chemoresistance in a wild-type p53-dependent manner. Notch1 appears to inhibit p53 through PI3K/Akt pathway and its inhibition reverses chemoresistance [13].

P53 is a powerful regulator of the cell cycle and is essential in preventing inappropriate cell proliferation and in maintaining genome integrity following genotoxic stress [14]. Loss of p53 function is commonly encountered in human cancer and restoration of p53 function leads to apoptosis in lymphomas [15]. In the context of the impact of apoptosis on therapeutic outcome, novel strategies to restore the apoptotic p53 pathway have been vigorously pursued [16,17]. Here, we have explored RES-induced apoptosis in T-cell acute lymphoblastic leukaemia and have attempted to elucidate the signalling pathways mediating the apoptotic effects of RES.

In order to investigate the cellular pathways involved in RES-induced apoptosis we analysed the effects of RES on Notch, p53, and Akt pathways. We found that RES reduces cell proliferation and induces apoptosis in MOLT-4 cells in a dose and time-dependent manner. We also found that RES activates the p53 pathway and inhibits the PI3K/Akt pathway and therefore activates Gsk-3β. RES is also able to reduce the level of Notch, possibly acting on Gsk.

2. Materials and methods

2.1. Reagents

RES (R5010), Chloroquine (C6628); MG132 (C2211) and Lithium Chloride (L4408) were from Sigma–Aldrich. Phospho-Akt (Ser473) (#9271), Gsk-β (#9332) and Phospho-Gsk-β (Ser9) (#9336) antibodies were from Cell Signaling Technology. Antibody against NOTCH-IC (sc-6014) was obtained from Santa Cruz Biotechnologies Inc. Antibodies against p21 (P1484), p53 (P5813) and Bax (B3428) were from Sigma–Aldrich. The antibody specific for phospho-Ser15-p53 was used [phospho-specific (Ser15) anti-p53 (PC 461) from Oncogene Research products].

2.2. Cell cultures

MOLT-4 human T-ALL cell line was maintained as previously reported [18] in 5% CO_2 atmosphere in complete RPMI-1640 medium supplemented with 10% heat inactivated foetal bovine serum and penicillin/streptomycin (Euroclone).

2.3. RT-PCR

Total RNA was extracted from cells using the Chomczynski and Sacchi method [19]. RNA samples were reverse-transcribed by M-MLV Reverse Transcriptase (Gibco BRL-Life Technologies) according to the manufacturer's instructions. PCR was performed in a 20 μl reaction mixture containing 2 μl of cDNA, 1× PCR Buffer II, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.25 units of AmpliTaq DNA polymerase (Perkin-Elmer Corporation). Reactions were amplified in a GeneAmp PCR System 9700 thermal cycler as previously reported [20]. Amplified cDNAs were separated by agarose gel electrophoresis in the presence of ethidium bromide (100 ng/ml). Results of electrophoresis were acquired by the image acquisition system EDAS 2400 (Kodak) and analysed by Kodak 1D image analysis system.

2.4. Western blot assay

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer. The lysates were centrifuged at 10,000 rpm for 10 min at 4 °C. Protein concentration was measured by Bio-Rad Protein Assay. Total proteins (60 μg/lane) were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were probed with the indicated antibodies and signals were detected with the enhanced chemiluminescence (ECL) kit (Amersham Biosciences). The westerns were routinely normalized using actin (Santa Cruz Biotechnology Inc.; SC-10731).

2.5. Apoptosis analysis by Annexin V & PI staining

Cells undergoing apoptosis were identified by Annexin V and propidium iodide staining (MBL) according to the manufacturer's instructions. Phosphatidylserine, which is

normally located on the cytoplasmic surface of cell membrane, is exposed on the cell surface upon induction of apoptosis. Annexin V binds to phosphatidylserine and is used to identify the earliest stages of apoptosis. PI, which does not enter cells with intact membranes, is used to distinguish between early apoptotic cells (Annexin V-positive) and late apoptotic or necrotic cells (Annexin V–PI-double positive). Briefly, 5×10^5 cells were washed once in PBS and resuspended in 500 μl of binding buffer 1× (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). PI (final concentration 2 μg/ml) and FITC-conjugated Annexin V (final concentration 0.8 μg/ml) were added; cell suspension was incubated for 5 min in the dark and analyzed by fluorescent microscopy.

3. Results

3.1. *Inhibition of proliferation and Induction of apoptosis by RES*

We first tested the effects of RES on viability of MOLT-4. The cells were treated once with different concentration of RES for 48 h and the fraction of viable cells was assessed by Trypan Blue staining. As shown in Fig. 1A RES induced a dose- and time-dependent decrease in cells number compared with cells incubated in medium alone.

To determine whether the decrease in cell viability was attributable to apoptosis, cells were stained with FITC conjugated Annexin V plus PI and evaluated by fluorescence microscopy at different time points. As illustrated in Fig. 1B, cells undergo dose-dependent apoptotic cell death in response to RES.

We then treated the cells with RES 64 μM for 72 h and measured the fraction of viable and apoptotic cells at different time points. RES induced a significant decrease of cell viability due to apoptotic cell-death (Fig. 2A and B).

These data confirm that RES is effective in reducing cell viability through the induction of apoptosis in the T-ALL line MOLT-4.

3.2. *RES activates p53 pathway*

In order to determine whether the activation of the p53 pathway was involved in RES-induced apoptosis, analyses of p53 and its down stream effectors were performed. A marked increase in p53 phosphorylation at Ser15, indicating p53 activation, occurred at 16 h after RES administration. The antibody specific for phospho-Ser15-p53 [phospho-specific (Ser15) anti-p53 (PC 461) from Oncogene Research products] was employed in this experiment (Fig. 3). The expression of Bax, a well known target of p53, increased during RES treatment from the start of the treatment. The increase in p53 activation was also accompanied by the induction of p21waf1 transcription at 24 h after RES administration which was sustained up to 72 h.

The increase in phosphorylated p53 and its down stream target gene products Bax and p21waf1 suggest the operation of a p53-mediated mechanism in MOLT-4 cells apoptosis during RES treatment.

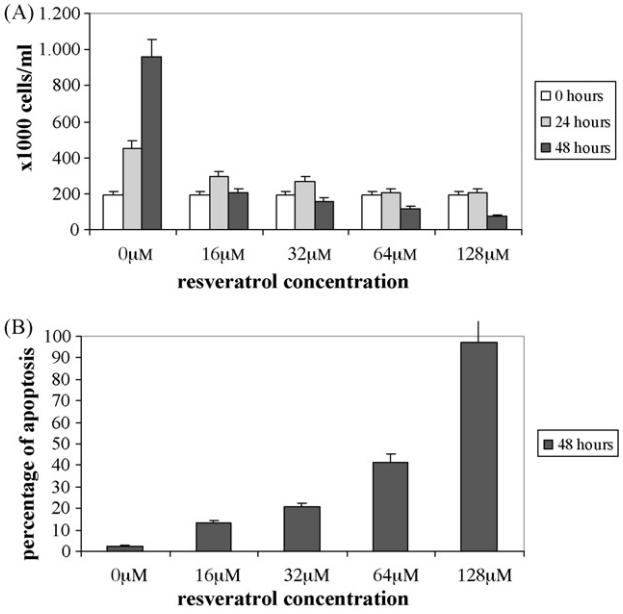


Fig. 1 – (A) Illustrates RES induced dose- and time-dependent decrease in cells number compared with cells incubated in medium alone. (B) Cells undergo dose-dependent apoptotic cell death in response to RES.

3.3. *RES inhibits the PI3K/Akt pathway*

We next investigated whether RES modulated the Akt pathway in MOLT-4 cells. As shown by the immunoblot analysis in Fig. 4, RES treatment resulted in an appreciable down regulation of the active form of Akt (phospho-Ser473-Akt) from very early time points without any changes in total Akt

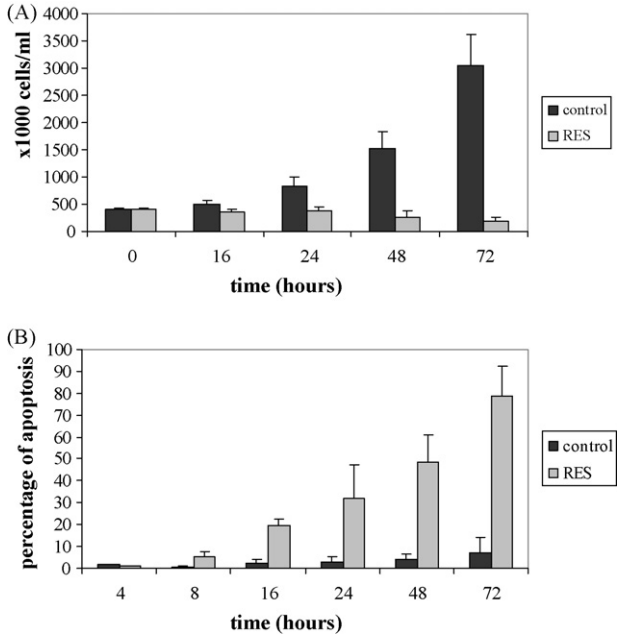


Fig. 2 – (A and B) RES induced decrease of cell viability due to apoptotic cell-death.

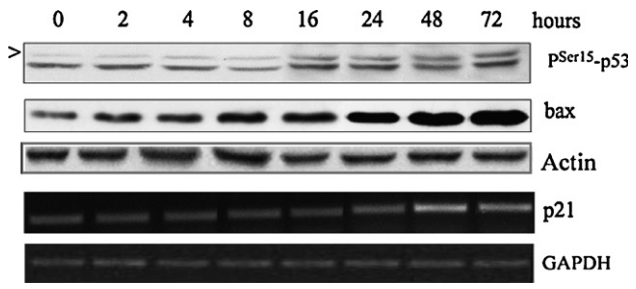
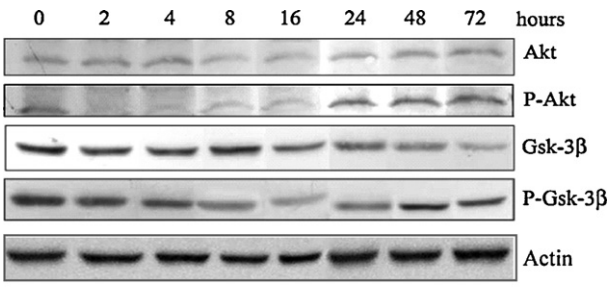


Fig. 3 – The expression of p53 and its downstream effectors. A marked increase in p53 phosphorylation at Ser15, indicating p53 activation, occurs at 16 h after RES administration together with Bax expression from starting point of RES treatment. The antibody specific for phospho-Ser15-p53 [phospho-specific (Ser15) anti-p53 (PC 461) from Oncogene Research products] was employed in this experiment. The increase in p53 activation was also accompanied by induction of p21waf1 transcription 24 h after RES treatment, which was maintained up to 72 h.



0h	2h	4h	8h	16h	24h	48h	72h
1	0,92	0,79	0,27	0,25	0,66	1,4	2

Fig. 4 – The down regulation of the active form of Akt (phospho-Ser473-Akt) from very early time points of RES treatment, but without significant changes in total Akt protein. RES also leads to a significant decrease Gsk-3β phosphorylated at Ser 9 in comparison with total Gsk-3β: a table shows the P-Gsk-3β/total Gsk-3β ratio.

protein. The P-Akt levels returned to basal levels after 16 h from the beginning of treatment. We next determined whether RES treatment was mediated by Gsk-3β activation. RES administration led to a decrease in Gsk-3β phosphorylation at Ser 9 from 8 h to 24 h, as indicated by P-Gsk-3β/total Gsk-3β ratio obtained through bands quantification (Fig. 4). This suggests the possible involvement of the Akt/Gsk-3β pathway in the antiproliferative/proapoptotic response to RES.

3.4. *RES inhibits the Notch pathway*

Notch signalling, which is deregulated in T-ALL, was also analysed. Our results show that escalating doses of RES led to a progressive decrease in Notch protein expression (Fig. 5A). The decrease in Notch protein levels was appreciable starting from 16 h after treatment with 64 μM RES (Fig. 5B). In order to determine whether the modulation of Notch was due to a transcriptional or post-transcriptional mechanism, RT-PCR was performed on total RNA extracted from MOLT-4 cells. As shown in Fig. 5C, RES treatment did not affect the levels of Notch RNA. We confirmed the inhibition of the Notch signalling by analysing the expression of preTCRα alpha and HES1, which are regulated by Notch. We found a decrease in the RNA levels of both genes starting from 24 h after RES treatment (Fig. 5C).

3.5. *Gsk-3β mediates the apoptotic effect of Resveratrol*

To further investigate the role played by the Akt/Gsk-3β pathway in the induction of apoptosis, we selectively inhibited Gsk-3β using lithium chloride (LiCl) during RES treatment. The cells were treated with 10 mM LiCl and 64 μM Resveratrol for 48 h and analysed for apoptotic cell death and phospho-Ser9-Gsk-3β. LiCl inhibited Gsk-3β by inducing an increase of the inactive form of the kinase (Fig. 6A). Gsk-3β inhibition resulted in a significant decrease in apoptotic cell death (Fig. 6B). These data suggest that activation of Gsk-3β plays an essential role in inducing apoptosis during RES treatment.

3.6. *Lithium chloride modulates notch pathway*

In order to determine the effect of LiCl on the other pathways that we found modulated during RES treatment, we performed a western-blot assay for NOTCH-IC. The cells were treated with 10 mM LiCl and 64 μM RES for 48 h and total proteins were extracted. As can be seen in Fig. 7, in the presence of RES, LiCl induced an increase in NOTCH-IC.

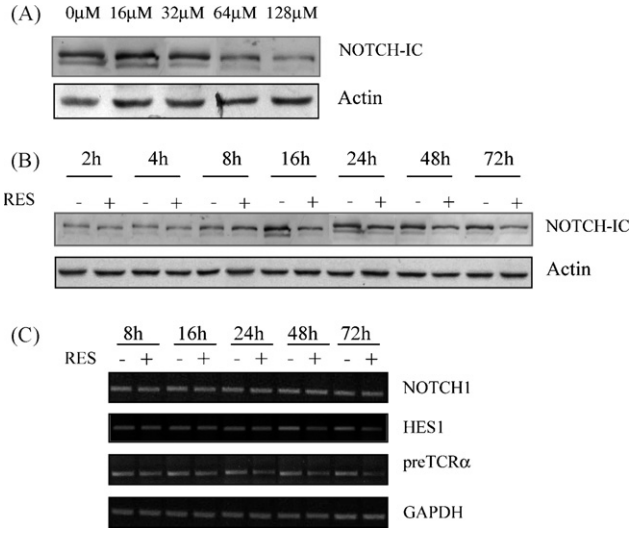


Fig. 5 – (A and B) The progressive decrease in Notch protein expression engendered by RES (A) without affecting Notch RNA levels (B). The corresponding loading control has also been shown in the figure. (C) Notch pathway by examining the expression of preTCRα and HES1. RNA levels of both targets decreased starting from 24 h after RES administration, thus confirming that RES could inhibit the Notch pathway.

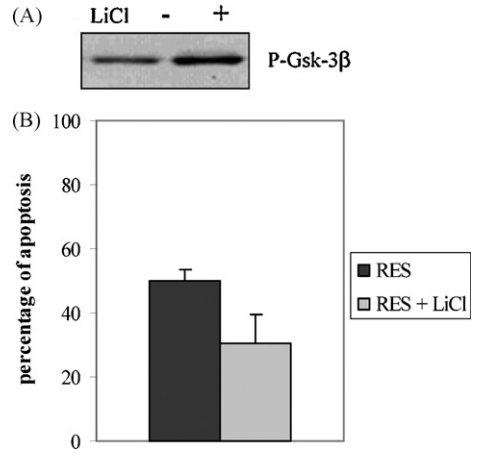


Fig. 6 – (A and B) Gsk-3β mediation of the apoptotic effect of Resveratrol. (A) LiCl induces an increase of the inactive form of the kinase. (B) Gsk-3β inhibition results in a decrease in apoptosis. Statistical analysis was performed by Student two-tail *t* and displays that apoptosis ratio variation between MOLT-4 cells treated with RES and RES + LiCl is significant at $P < 0.01$.

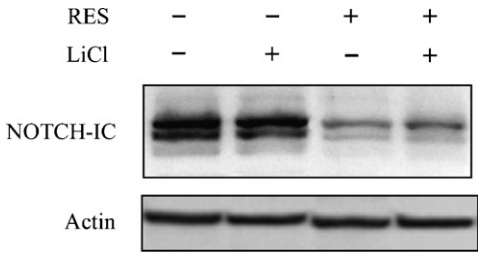


Fig. 7 – The increase of Notch1C, p53 and bax protein levels following LiCl treatment of cells. This figure, which includes the steady-state control, shows that Gsk-3β inhibition by LiCl reverses somewhat RES-induced Notch1 down regulation, suggesting a possible role of Gsk-3β in Notch1 regulation downstream AKT.

4. Discussion

Despite aggressive therapies, the resistance of many tumours to treatment constitutes a major problem in cancer therapy. Defects in the apoptosis programme that frequently characterises cancer cells may cause resistance. Particularly leukaemic cells invariably have abnormalities in one or more apoptotic pathways; this is a survival advantage for these cells over normal counterparts. Furthermore, abnormalities in the apoptotic response also play a role in the development of drug resistance by leukaemic cells. The induction of apoptosis is a key mechanism for most antitumour therapies, and chemoprevention is regarded as a promising strategy to control cancer [21,22]. Many natural or dietary substances have been shown to inhibit experimental carcinogenesis [23]. Much attention has been focused on RES, a natural phytoalexin, which exerts a wide array of biological effects, including anti-inflammatory, anti-proliferative and potential chemopreventive activity against human cancer [24]. Moreover, RES has been shown to suppress the growth of transformed cells also through induction of apoptosis [25,26]. RES exerts a pro-apoptotic effect in HL60 leukaemia cells as well as in T47D breast carcinoma cells but it does not affect human peripheral blood lymphocytes from healthy donors [27]. Patients with acute T-cell leukaemia have sometimes poor treatment outcome because of the intrinsic drug resistance to chemotherapy and protection from apoptosis, a common feature of multidrug-resistance of T-ALL [28].

We examined the human T-cell acute lymphoblastic leukaemia cell line (MOLT-4) to unravel the pathways of RES-induced apoptosis with the view to focus on the putative cancer chemopreventive effect of this product. We investigated the operation of three potential pathways associated with the induction of apoptosis by RES in a cell-specific manner.

The experiments described here show that in the experimental conditions employed RES induces both dose- and time-dependent cancer cell growth inhibition, and this antiproliferative effects on MOLT-4 cells, which in part, appears to be due to its ability to induce apoptotic cell death. Notch signalling is known to interact with Akt- and p53-mediated pathways, to promote cell survival [29]. Notch1, for instance, has been shown to inhibit p53-mediated apoptosis during leukaemogenesis [30] and in immortalised epithelial cells [31,32]. Notch 1 also promotes the survival of primary thymocytes via an Akt-dependent pathway [33].

The Notch signalling pathway is involved in the transduction of extracellular developmental signals to the nucleus, Notch1 signalling plays essential roles in the thymus as regulator of T cell lineage commitment and thymocyte development [34,35]. This pathway is constitutively activated by chromosomal translocation or mutations rendering these events strongly oncogenic [11,12]. Indeed, these occur in over 50% of human T-ALL [12,36]. Moreover, Notch signalling is known to inhibit apoptosis [37]. Our data show that RES is able to reduce the level of Notch; the effects could be mediated by Gsk. Notch1 targets in T lineage cells include HES1, Deltex1, Notch3, nRARP (Notch regulated ankyrin-repeat protein) and pTalpha [38]. RES down regulated HES1 and pTalpha, which confirms the participation of Notch signalling in the induction of apoptosis. This is the first report of Notch involvement in this process.

With this demonstration it became imperative to know if there was potential reciprocal regulation of Notch signalling during RES induced inhibition of apoptosis. Activation of p53 directs cells to undergo cell cycle arrest and/or apoptosis. p53 is the most frequently inactivated gene in human malignancies and its inactivation is beneficial for tumour survival. It follows that the suppression of p53 by Notch is an important event in the development of leukaemia and the activation of p53 mediates regression of disease. We found that RES treatment activates the p53 pathway increasing the expression of Bax and p21.

The second apoptosis pathway, which we show here to interact with Notch signalling, is the PI3K/Akt system. Akt signalling figures prominently in tumorigenesis. Hyperactivation of Akt kinases is a common event in many human cancers [39,40] and this results in tumour cell survival and enhanced resistance to apoptosis [41,42]. Akt signalling

disrupts activated Notch1 function and thus appears to be critical for apoptosis to occur [43]. RES decreased Akt levels in a time-dependent manner indicating that these reduced Akt levels contributed to the apoptosis of MOLT-4 cells. The mediation of Akt and PTEN in RES effect has been described before [44–46]. It is evident from our work that RES inhibits the PI3K/Akt pathway and activates Gsk-3β. Indeed, activation of Gsk-3β plays an essential role in RES induced apoptosis as manifested by the effect of LiCl on the three pathways, which were modulated by RES. When viewed in the background of the report that Notch1 inhibits p53-mediated apoptosis through PI3K/Akt pathway and its inhibition reverses chemoresistance, our data are clearly suggestive of a linkup or interaction between these three signalling pathways in response to RES treatment.

5. Concluding remarks

Here we demonstrate unequivocally that RES induces apoptosis by inhibiting the Notch pathway and markedly influencing the operation of the interacting apoptosis pathways mediated by p53 and PI3K/Akt. Thus we have identified the potentially significant signalling pathways influenced by RES that could lead to the identification of the targets of RES-induced apoptosis and growth control. Our data do advocate, in conformity with the views expressed by other authors, probing the potential of RES as a chemopreventive agent.

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