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ORIGINAL ARTICLE

Antiproliferative effects of rapamycin as a single agent and in combination with carboplatin and paclitaxel in head and neck cancer cell lines

Nasredine Aissat · Christophe Le Tourneau · Aïda Ghoul · Maria Serova · Ivan Bieche · François Lokiec · Eric Raymond · Sandrine Faivre

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Abstract
Purpose Recent data suggested that combining targeted therapies with chemotherapy may counteract drug resistance. Activation of the PI3K/AKT/mTOR pathway downstream to kinase receptors, such as EGFR, was found in 57–81% of head and neck squamous cell carcinoma (HNSCC), and was eventually associated with a loss of PTEN function. mTOR was shown to modulate cell proliferation, apoptosis, invasion, and angiogenesis. This study aimed to evaluate molecular and cellular effects of rapamycin in a panel of cell lines either as single agent or in combination with cytotoxics commonly used in HNSCC.
Methods Antiproliferative effects of rapamycin, carboplatin, and paclitaxel were evaluated in a panel of three HNSCC cell lines (SCC61, SQ20B and HEP2). Cells were exposed to rapamycin for 48 h, to carboplatin for 48 h, or to paclitaxel for 24 h. Antiproliferative effects of simultaneous and sequential rapamycin-based combinations were studied using MTT assay and median effect plot analysis. Cell cycle effects were analysed using flow cytometry.
Results Rapamycin induced concentration dependent antiproliferative effects in HNSCC cell lines with IC_{50} of 5 ± 1, 12 ± 2 and 20 ± 2 μM in SCC61, SQ20B, and HEP2 cells, respectively. Higher antiproliferative effects were observed in SCC61 cells overexpressing NOXA and cyclin D1 than in HEP2 that overexpressed MDR1 and BCL2. In our panel, antiproliferative effects of rapamycin were associated with G0/G1 cell cycle accumulation and apoptosis induction, at concentrations ranging 3–30 μM. Combinations of rapamycin with paclitaxel and carboplatin displayed synergistic and additive effects. Synergistic effects were observed with paclitaxel in SQ20B and HEP2 cells and with carboplatin in SQ20B cells, when cells were exposed to cytotoxics prior to rapamycin.
Conclusion Our results show that rapamycin displays antiproliferative effects and induces apoptosis in HNSCC cell lines, cellular effects being more potent in cells that do not express BCL2 and MDR1. Additive and synergistic effects were observed when rapamycin was combined with carboplatin and paclitaxel.

Keywords mTOR · PI3K/AKT pathway · Cytotoxicity · Apoptosis

N. Aissat · C. Le Tourneau · A. Ghoul · M. Serova · E. Raymond · S. Faivre
Department of Experimental Pharmacology (RayLab) and Medical Oncology, Beaujon University Hospital, AP-HP, Clichy, France

I. Bieche
Laboratory of Molecular Genetics, Beaujon University Hospital, Clichy, France

F. Lokiec
Department of Clinical Pharmacology, Centre René Huguenin, Saint-Cloud, France

S. Faivre (✉)
Service Inter-Hospitalier de Cancérologie Bichat-Beaujon, 100, boulevard du général Leclerc, 92110 Clichy, France
e-mail: sandrine.faivre@bjn.aphp.fr

Introduction

Recent advances in the understanding of molecular events underlying initiation, progression, and occurrence of metastases have led to the development of molecular targeted therapies of cancer. For example, it has been

shown that the epidermal growth factor receptor (EGFR) was overexpressed in most of HNSCC and was associated with a poor prognosis [1, 2]. Cetuximab, a monoclonal antibody directed against EGFR, has been recently approved in combination with radiation therapy in patients with unresectable HNSCC, and in combination with cisplatin after cisplatin failure in patients with recurrent or metastatic HNSCC. In addition, cumulative evidences have suggested that the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR (mammalian target of rapamycin) signalling pathway plays a key role in the regulation of many cellular processes, including cell proliferation, survival, and apoptosis [3]. mTOR is a 289-kDa serine/threonine kinase belonging to the phosphoinositide kinase-related family that may be activated by EGFR in 57–81% of patients with HNSCC [4, 5]. The mTOR pathway controls several of fundamental cell functions, such as translation, initiation, proteins stability, ribosomal synthesis, thereby playing a central role in the regulation of cell growth, proliferation and survival [5, 6]. mTOR activation results in the phosphorylation of the ribosomal p70S6K (S6K1) protein and the 4E-BP1 translational repressor [7]. mTOR pathway appears to be particularly overactivated in cell lines with activated AKT [8].

Rapamycin is a lipophilic macrolide antibiotic isolated from the soil bacterium *Streptomyces hygroscopicus* in the mid 1970s [9] that was initially developed as an immunosuppressant and was subsequently found to display potent antitumour activity in a variety of solid tumours. Rapamycin binds the 12-kDa immunophilin FK506-binding protein (FKBP12) and forms a complex that inhibits mTOR. The inhibition of mTOR leads to dephosphorylation of S6K1 and 4E-BP1, which in turn, inhibits translation and blocks cell cycle in G1 phase [10]. Based on data obtained with rapamycin, several rapamycin analogs, such as CCI-779, AP 23573 and RAD001, are currently developed as anticancer agents.

While the PI3K/AKT/mTOR signalling pathway was shown to be frequently activated in HNSCC, the effects of mTOR inhibitors either alone or in combination with other anticancer agents frequently used in this disease was not yet explored. Other studies reported that rapamycin and its analogs may increase the antitumour activity of a variety of cytotoxic agents, including cisplatin, doxorubicin, paclitaxel, topotecan and mitoxantrone [11–14]. In this study, we evaluated the cellular and molecular effects of rapamycin as a single agent or in combinations with carboplatin or paclitaxel in a panel of HNSCC cell lines. Our results suggest that rapamycin and rapamycin-based combinations may warrant further clinical investigations in patients with HNSCC.

Materials and methods

Cell lines

Human SCC61, SQ20B, and HEP2 HNSCC cell lines were obtained as a gift from Pr Jean Bourhis, Institut Gustave Roussy (Villejuif, France). SCC61 cells were known to be wild-type p53 [15], while SQ20B and HEP2 cells were known to have p53 mutations [16, 17]. Cells were cultured in RPMI media containing 10% foetal bovine serum, 2 mM L-Glutamine and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C.

Western blot analysis

To characterize the HNSCC cell lines, protein levels of PTEN, AKT and BCL2 were determined by western blot. Cells were lysed in buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triron X-100, 2 mM vanadate, 100 mM NaF, and 0.40 mg/ml phenylmethylsulfonyl fluoride. Equal amounts of protein (25 µg/lane) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in 0.05% Tween 20/phosphate-buffered saline and then incubated with the primary antibody overnight. Membranes were then washed and incubated with the secondary antibody conjugated to horseradish peroxidase. Bands were visualized by using the enhanced chemiluminescence (ECL) western blotting detection system. The following primary antibodies were used: anti-PTEN, and anti-AKT (Cell Signalling, Saint-Quentin-en-Yvellines, France), anti-BCL2 (Sigma, France). All antibodies were used at a 1:1000 dilution.

Real-time RT-PCR

The HNSCC cell lines were characterized for expression product of genes implicated in apoptosis (BCL2 and NOXA), cell proliferation (EGFR and IRS1), cell cycle (Cyclin D1) and drug resistance (MDR1). The theoretical and practical aspects of real-time quantitative RT-PCR using the ABI prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) have been described in detail elsewhere [18]. Total RNA was reverse-transcribed before real-time PCR amplification. The transcripts of the gene coding for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIID) were used as the endogenous RNA control, and each sample was normalized on the basis of its TBP content. Results were expressed as *N*-fold difference in target gene expression relative on the TBP gene. The thermal cycling conditions comprised in initial denaturation step at 95°C for 10 min and 50 cycle at 95°C for 15 s and 65°C for 1 min. Experiments were performed in duplicates for each data.

Cytotoxicity assays

Antiproliferative effects of rapamycin, paclitaxel and carboplatin were evaluated using a modified tetrazolium salt, MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma, Saint-Quentin Fallavier, France). Cell viability was detected by the colorimetric conversion of the yellow, water-soluble MTT, into purple, water insoluble formazan. This reaction is catalysed by mitochondrial dehydrogenases and is used to estimate the relative number of viable cells. Three thousand viable cells were plated in 100 µl of growth media in 96 well plates. After 24 h, cells were treated with different concentrations of rapamycin, paclitaxel or carboplatin for 24 and 48 h and post-incubated for 24 h in drug-free medium. MTT was then added at a concentration of 0.4 mg/ml to each well and incubated during 4 h at 37°C. The media was then removed, reduced MTT product was solubilized by adding 100 µM DMSO (Sigma, France). The absorbance was measured at 560 nm using a microplate reader (EX, Thermo, France). Experiments were performed in triplicates.

Combination study

For the evaluation of antiproliferative effects of rapamycin in combination with carboplatin or paclitaxel, three schedules were performed: (1) rapamycin for 48 h followed by carboplatin for 48 h or paclitaxel for 24 h, (2) carboplatin or paclitaxel followed by rapamycin for 24 h, (3) concomitant incubation of rapamycin with carboplatin or paclitaxel during 48 h. HNSCC cell viability was then determined by MTT assay. Experiments were performed in triplicates.

Median-effect plot

Rapamycin was combined with each chemotherapy agent (carboplatin or paclitaxel) at different concentrations of each drug (IC_{20}, IC_{40}, IC_{60} and IC_{80}). Concentrations were determined on the basis of the dose-response curves obtained using standard MTT assay. Combination index (CI) values were calculated for different dose-effect levels on the basis of parameters derived from median-effect plot analysis with rapamycin, carboplatin or paclitaxel alone, and the combination of rapamycin with carboplatin or paclitaxel at fixed molar ratio. Median-effect plot analysis and calculation of CI were analysed by the method of Chou and Talalay [19] using the Calcusyn software (Biosoft, Cambridge, UK). CI values of <1 indicate synergy, a value of 1 indicates additive effects and a value of >1 indicates antagonism. Variability between experiments led us to consider that CI values ranging from 0.8 to 1.2 mainly represent additive effects. Thereby, calculation of a CI below 0.8 was considered as an indication of synergy, values above 1.2 were considered as antagonism, and values ranging 0.8–1.2 considered as an indication of additive effects.

Cell cycle and apoptosis analysis

Cell cycle distribution and apoptosis were analysed by flow cytometry. To evaluate the effects on cell cycle, SCC61, SQ20B, and HEP2 cells were plated in tissue culture dishes in the presence or absence respectively of 7, 10 and 20 µM rapamycin for 48 h. After 48 h incubation, both adherent and detached cells were harvested. Cells were then washed once and fixed in 70% ethanol during 24 h at 4°C. Cells were then centrifuged, washed in PBS and incubated with 1 µg/ml RNAse A during 30 min at 37°C and with 12.5 µg/ml propidium iodide (PI) in the dark. DNA content was analysed by a FACScan flow cytometer (Becton Dickinson, NJ, USA).

Apoptosis was determined using Annexin V-FITC Apoptosis Detection Kit (Sigma). One of the early events in apoptosis, the translocation of phosphatidylserine from the inner layer of the plasma membrane to the outer layer, can be detected by binding of Annexin V to the cell surface. Cells were treated with rapamycin during 48 h. For each sample, cells were trypsinized and rinsed in binding buffer, then resuspended in 200 µl of binding buffer, containing 5 µl of Annexin V and 10 µl of PI. Cells were incubated during 15 min at room temperature in the dark and then analysed by flow cytometry. Experiments were performed in duplicates.

Statistical analysis

For statistical analysis and graphs, Prism software (GraphPad, San Diego, USA) was used. Experiments were performed in duplicates or triplicates. Means and standard deviations were compared using Student's *t* test (two-sided *P* value).

Results

Molecular characterization of the HNSCC cell lines

We first characterized our cell lines for the expression of key regulators of mTOR signalling and drug-induced apoptosis. The protein expression of PTEN was similar across cell lines, while AKT expression was higher in SCC61 and SQ20B than in HEP2 cancer cells (Fig. 1a). Similarly, EGFR and IRS1 were also expressed in all cell lines, although higher levels were detected in SQ20B cells. As shown in Fig. 1b, BCL2 expression was higher in HEP2 cells, than that in SQ20B and SCC61 cells both at protein and mRNA levels. The mRNA expression level of the

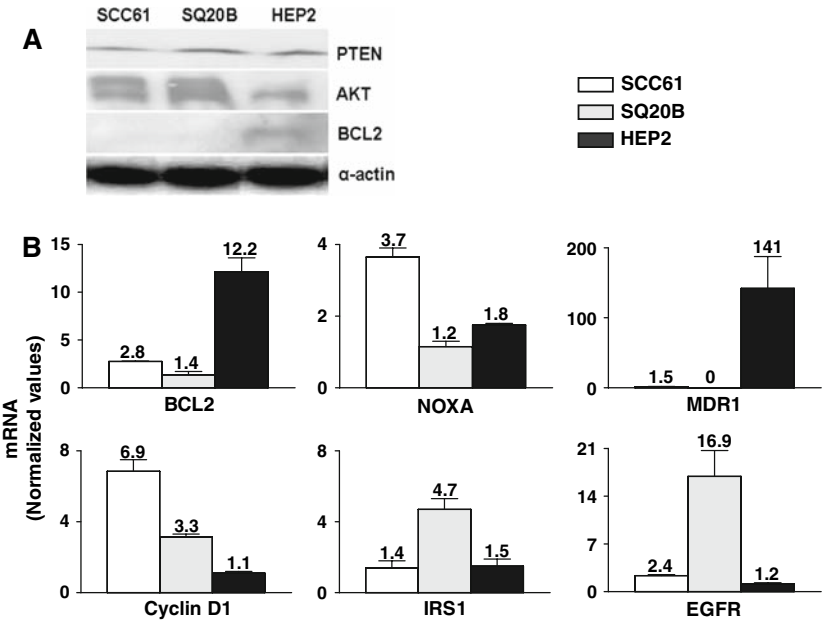


Fig. 1 **a** Western blot analysis of PTEN, BCL2 and AKT proteins expression in HNSCC cell lines. Western blot was representative of two separate experiments. **b** Quantitative RT-PCR analysis of BCL2, NOXA, MDR1, EGFR, IRS1, and Cyclin D1 mRNA in HNSCC cell lines. Data are means of two independent experiments performed in duplicates

pro-apoptotic gene NOXA was higher in SCC61 than that in SQ20B and HEP2 cells. In addition, MDR1 mRNA was overexpressed in HEP2 cell line, and almost not detectable in SCC61 and SQ20B cells. Cyclin D1 mRNA expression was higher in SCC61 than that in SQ20B and HEP2 cells. In summary, mTOR signalling appeared to be activated at different levels in all our HNSCC cell lines. Interestingly, SCC61 appeared to retain functional apoptotic pathway, and activated cyclin D1, while HEP2 expressed high level of BCL2 and MDR1 that are characteristics frequently associated with resistance to anticancer agents.

Antiproliferative effects of rapamycin as a single agent

The antiproliferative effect of rapamycin was determined using concentrations ranging 0.001–100 μM for 48 h (Fig. 2). After 48 h exposure, rapamycin induced concentration-dependent antiproliferative effects in HNSCC cell lines with IC_{50} of 5 ± 1, 12 ± 2 and 20 ± 2 μM in SCC61, SQ20B, and HEP2 cells, respectively. Interestingly, HEP2 that express a high level of BCL2 was fourfold more resistant to rapamycin than SCC61 that displays a high level of cyclin D1 and do not express BCL2.

Antiproliferative effects of carboplatin and paclitaxel

Carboplatin and paclitaxel displayed antiproliferative effects with IC_{50} of 7 ± 2, 11 ± 3 and 20 ± 1 μM for carboplatin, and 0.0005 ± 0.0001, 0.5 ± 0.2 and 1.5 ± 0.3 μM for paclitaxel in SCC61, SQ20B and HEP2, respectively. Consistent with results obtained with rapamycin, unfunctional apoptosis and MDR1 expression in HEP2 translated in low sensitivity to carboplatin and paclitaxel. Conversely,

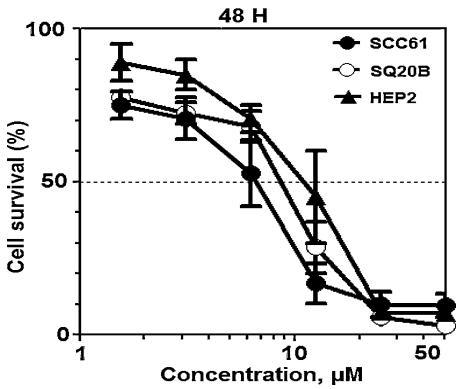


Fig. 2 Antiproliferative effects of rapamycin in HNSCC cell lines after 24 and 48 h of treatment. The data represent the mean values of normalized MTT results from three experiments

SCC61 that expresses NOXA but neither BCL2 nor MDR1 appeared to be more sensitive to cytotoxic agents.

Effects on cell cycle and apoptosis

To better understand the effect of rapamycin on cell growth, we exposed cells to rapamycin and evaluated changes in the cell cycle distribution after 48 h. Rapamycin induced a marked increase ($P < 0.05$) in the number of cells in Sub-G1 phase of cell cycle in SCC61 and SQ20B cell lines (Fig. 3). We further examined the capacity of rapamycin of inducing apoptosis. Cell cycle analysis suggested occurrence of apoptosis that was supported using Annexin V staining. Although direct comparison cannot be made between these two methods, our data are consistent with apoptosis induction. As shown in Fig. 4, HEP2 cells appeared to be more resistant to apoptosis induction than

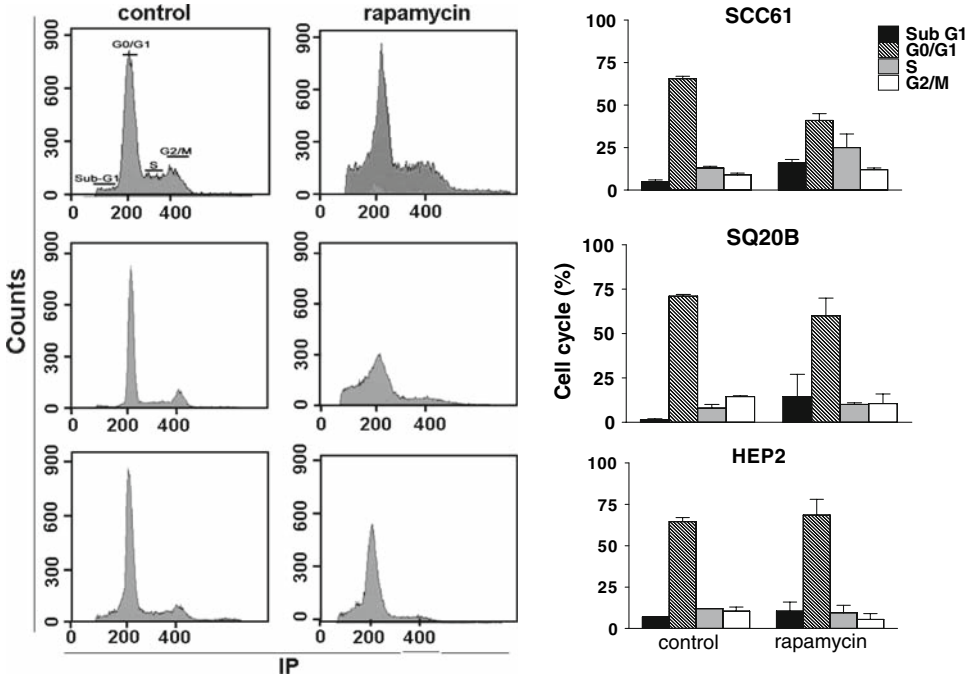


Fig. 3 Effects of rapamycin on cell cycle distribution in HNSCC cell lines. Distribution of cell cycle of SCC61, SQ20B and HEP2 cells was analysed by flow cytometry after 48 h treatment with 7, 10 and 20 μM rapamycin respectively. Percentages of cells in different phase of cell cycle are shown in histogram for each cell line

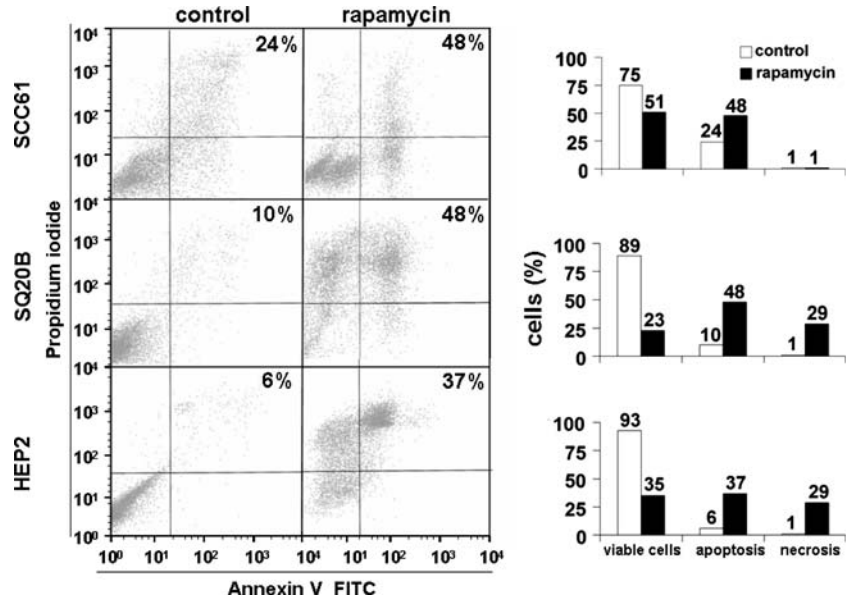
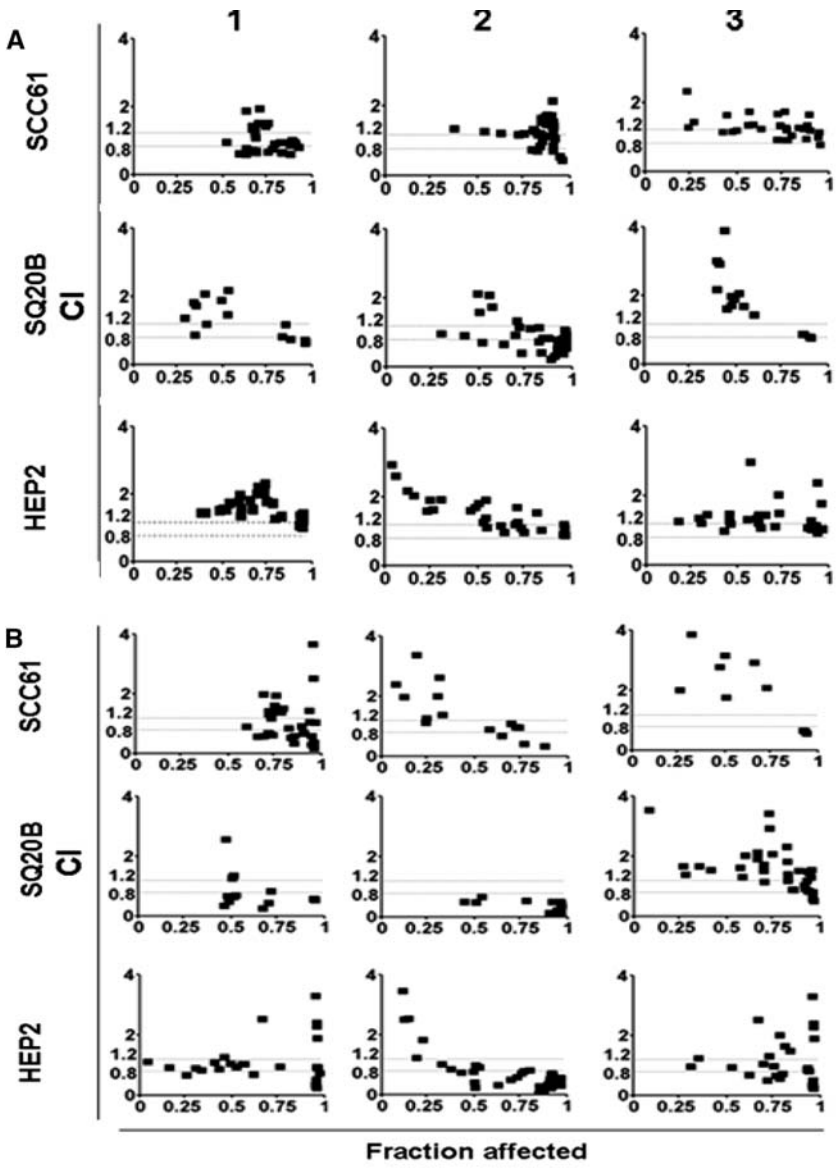


Fig. 4 Apoptosis in SCC61, SQ20B and HEP2 cells was monitored after after 24 h treatment with 7, 10 and 20 μM rapamycin, respectively, using flow cytometry (Annexin V-FITC). Percents of viable cells, apoptotic and necrotic cells are shown in histogram for each cell line

SQ20B and SCC61 cells. In addition, rapamycin was also shown to be capable of inducing a high level of necrosis in HEP2 and SQ20B cells (29%) as compared to SCC61 cell (1%). These results suggest that rapamycin may induce apoptosis in HNSCC cells, higher level being observed in cells that express cyclin D1 and lack BCL2 expression.

Rapamycin combinations with carboplatin or paclitaxel

Results of combination of rapamycin with carboplatin and paclitaxel are presented in Fig. 5. Regardless of the sequence, carboplatin combinations displayed additive effects with rapamycin and seemed to be able achieving synergism in SQ20B cells. When rapamycin was combined with paclitaxel, synergistic and additive effects were observed in all cell lines. In SQ20B and HEP2 cells, synergistic effects were more pronounced when cells were exposed to paclitaxel prior to rapamycin. In SCC61 cells, rapamycin showed at least additive effects with paclitaxel. Taken together, these results suggest that rapamycin enhances paclitaxel-induced cytotoxicity in a sequence-dependent manner. Considering the potential effects of rapamycin on cell cycle distribution that may explain the sequence dependency of the effects with cytotoxic, we evaluated cell cycle effects of rapamycin-based combinations. As shown in Fig. 6, HEP2 cells that display the



C

Mean of combination index (mCI)						
Combination schedules	rap-car	car-rap	rap+car	rap-pac	pac-rap	rap+pac
SCC61	1.01	1.17	1.08	0.95	1.6	1.21
SQ20B	1.28	0.73	1.43	0.63	0.52	1.11
HEP2	1.53	1.45	1.6	0.91	0.73	1.16

Fig. 5 Antiproliferative effects of rapamycin in combination with carboplatin (*C*) or paclitaxel (*P*) in HNSCC cell lines. **a** Combination of rapamycin with carboplatin; **b** combination of rapamycin with paclitaxel. Several schedules were investigated: (1) rapamycin followed by *C* or *P*, (2) *C* or *P* followed by rapamycin, (3) concomitant incubation of rapamycin with *C* or *P*. **c** The table represents the mean of CI (*mCI*) for each sequence of combination calculated for Fa from 0.25 to 0.75. *Fa* fraction affected; *CI* combination index

highest level of resistance to rapamycin, paclitaxel and carboplatin, were blocked in G0/G1 phase of cell cycle when exposed to 20 μM rapamycin with a number of cells undergoing apoptosis. When cells were exposed to rapamycin prior to paclitaxel, cell cycle distribution was similar to that of paclitaxel alone. Interestingly, exposure to paclitaxel prior to rapamycin resulted in an increase level of cells in Sub-G1 phase, suggesting that this sequence of the combination may be capable of enhancing apoptosis in this cell line. Similar results were observed with SQ20B cells exposed to rapamycin and paclitaxel (data not shown).

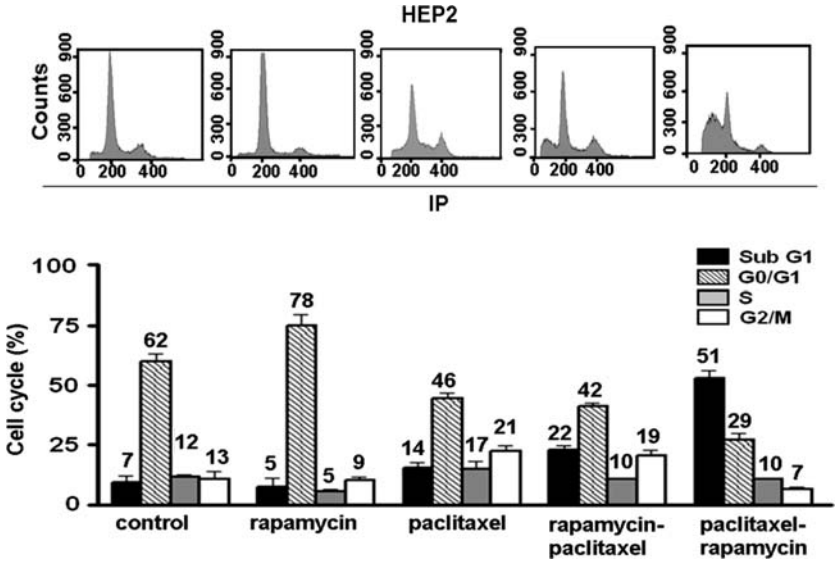


Fig. 6 Effects of 20 μM rapamycin in combination with 0.5 μM paclitaxel on cell cycle in HEP2 cell line. Percentages of cells in different phase of cell cycle are shown in histogram

Discussion

Current advances in the understanding how aberrant functions of signalling networks contribute to cancer cell growth can now be exploited for the identification of novel mechanism-based anticancer treatments. The PI3K/AKT/mTOR signalling pathway plays a major role in cell proliferation and cell cycle regulation. A number of recent data suggest that rapamycin, apart from its immunosuppressive activities, also exhibits potent anti-neoplastic effects in various tumours [20]. A number of previous and more recent data suggest that, depending on the cell type analysed, rapamycin inhibits cell growth through induction of cell cycle arrest and/or induction of apoptosis [21].

It was recently shown in literature, that amplification or overexpression of EGFR occurs in human HNSCC and was associated with resistance to chemotherapeutic agents such as paclitaxel [22, 23]. This was also observed in our study since SQ20B cells which exhibits the highest level of EGFR expression, were also most resistant to paclitaxel than HEP2 and SCC61 cells. Potential mechanisms of the resistance to cytotoxic agents may be related to the activation of cell signalling pathways that stimulate the cellular proliferation and survival, such as the PI3K/AKT/mTOR signalling pathway. To test whether rapamycin may counteract the activation of mTOR as a survival signal in resistance to paclitaxel and carboplatin, we investigated the antiproliferative effect of rapamycin in combination with those cytotoxics in HNSCC. Additive and synergistic effects that seem to be sequence-dependent were observed when rapamycin was associated with carboplatin or paclitaxel. Sequence dependency may be related to changes in cell cycle distribution after exposure to rapamycin. Other investigations have reported similar results using breast cancer cell lines, showing synergistic effect when rapamycin was administered after carboplatin or paclitaxel [23]. Interestingly, rapamycin increases the sensitivity to paclitaxel of SQ20B cells that overexpress EGFR, suggesting pharmacological benefit of investigating this combination in the clinic.

Taken together, our results suggest that rapamycin was able to potentiate the antiproliferative effect of carboplatin in SQ20B cell line, as well as of paclitaxel in HEP2 and SQ20B cell lines. These synergistic effects observed in the combinations of rapamycin with paclitaxel or carboplatin may be explained, at least in part, by the inhibition of the protein synthesis following the inactivation of mTOR by rapamycin that would prevent the cells from synthesis of new proteins required to repair alterated DNA. S6K1 has been found to phosphorylate and inactivate the pro-apoptotic molecule BAD, a process inhibited by rapamycin [24]. Interestingly, antitumour effects of both paclitaxel and the platinum agent cisplatin have also been proposed to be mediated, at least in part, by inhibition of S6K1 [25, 26]. Recent studies have shown that BCL2 overexpression leads to resistance of paclitaxel and blocks paclitaxel-induced apoptosis [27]. In addition, downregulation of BCL2 was observed in case of carboplatin-induced apoptosis [28].

Our results showed that antiproliferative effects may be observed in HNSCC cell lines at micromolecular concentrations that may readily be obtained using oral rapamycin in clinical trials [29, 30]. Interestingly, sensitivity of SCC61 and SQ20B cells may be explained, at least in part, by a low level of expression of anti-apoptotic factors such as BCL2 and high level of pro-apoptotic factors such as NOXA and cyclin D1. Conversely, HEP2 cells that express BCL2 and MDR1 were about fivefold more resistant to rapamycin than SCC61. Moreover, our findings in HNSCC cancer cells were similar to that obtained in studies using human ovarian cancer cell lines resistant to both

rapamycin and RAD001, which overexpress the anti-apoptotic gene BCL2 [31]. These data suggest that expression of BCL2 prevent cells to undergo apoptosis. This was previously shown by our group showing that downexpression of BCL2 using antisens oligonucleotides against BCL2 was capable of restoring apoptosis induction in ovarian cancer cells [31]. Importantly, BCL2 was also shown to inhibit apoptosis induced by cisplatin, carboplatin and paclitaxel [27, 28], making HNSCC that express BCL2 resistant not only to rapamycin but also to carboplatin and paclitaxel in cultured experiments. In addition, previous studies in other cancer cell lines have also shown that cyclin D1 expression, which is regulated by a cap-dependent translation, was decreased in rapamycin-sensitive breast cancer cells MCF-7 and MDA-MB-468, but not in any of the rapamycin-resistant breast cancer cell lines [32]. In our study and other previously published data, rapamycin was associated with a decrease level of cyclin D1 that prevent cell to progress in S-phase of cell cycle, leading to the accumulation of cells in G0/G1. Furthermore, the higher baseline expression of cyclin D1 in the rapamycin-sensitive cells (SCC61) suggests that cyclin D1 plays an important role in the proliferation of these cells and sensitivity to rapamycin. The resistance of HEP2 cells to rapamycin may all be explained by the higher expression of MDR1. These results are consistent with recent studies, which show that both CCI-779 and rapamycin have no antitumour activity in P-gp1 or MRP1 overexpressing cell lines [33].

In summary, our results showed that rapamycin used as single agent displays antiproliferative effects and induces apoptosis in HNSCC cell lines. Cellular effects were more potent in cells that express NOXA and cyclin D1 than in cells that express BCL2. Moreover, rapamycin potentiates the cytotoxicity of selected chemotherapeutic agents, especially paclitaxel. Our in vitro data suggest that this effect may be sequence- and cell line-dependent. From a clinical perspective, rapamycin-based combination therapies may be especially effective in appropriately selected patients. This study strongly suggests to further investigate the antitumour effects of rapamycin (and rapamycin derivatives) as single agent and in combination with chemotherapy in clinical trials.

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