



Zoledronic acid determines S-phase arrest but fails to induce apoptosis in cholangiocarcinoma cells

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ABSTRACT

Cholangiocarcinoma is the second most common primary hepatic neoplasia and the only curative therapy is surgical resection or liver transplantation. Biphosphonates (BPs) are an emerging class of drugs widely used to treat bone diseases and also appear to possess direct antitumor activity. In two human cholangiocarcinoma cell lines (TFK-1 and EGI-1) we investigated, for the first time, the activity of zoledronic acid by determining proliferation, cell cycle analysis and apoptosis.

The results obtained indicate that zoledronic acid induces cell-narrowing and growth inhibition, both reversed by 25 μ M GGOH, and significantly affects the colony-forming ability of these cells. The inhibition by zoledronic acid of Rap1A prenylation was reversed in cell co-treated with GGOH. At 10–50 μ M zoledronic acid exerted an S-phase cell cycle arrest which was confirmed by changes in the level of cyclins and of regulators p27^{KIP1} and pRb. Interestingly, the expression level of cyclin A (putative S-phase marker) shows a dose-dependent increment in contrast to the decrement of cyclin D1 (putative G1 phase marker). However, neither hypodiploid cells nor cleaved PARP or caspase-3 was detected. The lack of TP53 or loss of its function, the large constitutive expressions of anti-apoptotic proteins Bcl-xL and HSP27 together with the low level of the pro-apoptotic Bax are the likely factors which protect cells from apoptosis.

In conclusion, our study indicates that zoledronic acid induces S-phase arrest and cell-narrowing, both reversed by GGOH and, by changing the delicate balance between pro- and anti-apoptotic proteins, allows survival of cholangiocarcinoma cells.

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1. Introduction

Cholangiocarcinoma is a malignant tumor that derives from bile duct epithelium and is classified anatomically into intra- and extra-hepatic cholangiocarcinoma. The incidence and the mortality rates of cholangiocarcinoma are increasing worldwide [1]. At present, complete resection is the only way to cure this disease, but at the time of diagnoses more than 60% of patients have occult metastases or an advanced local disease which preclude any curative resection. Conventional anticancer treatments, such as chemotherapy or radiotherapy have minimal impact on patients survival with unresectable tumor [2–5]. Up to now, the factors responsible for this relative unresponsiveness have not been clearly understood. In general, the efficacy of conventional anticancer therapies is strongly dependent on their ability to

initiate programmed cell death (apoptosis) in cancer cells [6–8]. Therefore, cancer cells that have evolved ways to circumvent apoptosis become resistant, providing an obstacle to effective treatments [9,10].

Previous researchers have shown that zoledronic acid (ZOL), third generation of biphosphonates (BPs), exerts powerful anti-tumoral activity [11] in several human neoplasms [11] such as human myeloma [12], breast [13] and prostate [14,15] cancers. BPs are currently the most important class of inhibitors of osteoclast-mediated bone resorption and are used extensively for the treatment of skeletal diseases, such as Paget's disease, postmenopausal osteoporosis, and tumor-induced osteolysis [16,17]. The antiproliferative effect of pamidronate in human and rat osteosarcoma cells has recently been demonstrated *in vitro* [18,19]. BPs can be divided into two distinct pharmacological classes based on their molecular mechanisms of action [20]. Non-nitrogen-containing BPs, such as clodronate and etidronate, are metabolized intracellularly as cytotoxic analogues of ATP and thus inhibit osteoclast activity [21]. In contrast, the newer nitrogen-containing BPs such as alendronate, residronate, pamidronate, and ZOL inhibit

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cell proliferation and induce apoptosis in osteoclasts [22] by inhibiting farnesyl-PP-synthase of the mevalonate synthesis, a biosynthetic pathway responsible for the production of cholesterol and isoprenoid lipids, particularly farnesyl- and geranylpyrophosphates. These are required for the posttranslational modification (prenylation) of small GTP proteins (such as Rho, Ras, Rac) that play crucial roles in signaling pathways controlling cell growth. The antiproliferative effect of ZOL was described in several tumor cell lines and it was likely due to cell cycle distribution change by accumulating and arresting cells in the S-phase [23–27]. This arrest was coupled to changing levels of cyclins and cell cycle regulators, and the S-phase arrest was associated with typical (caspase-dependent) [25,27,28] or atypical (caspase-independent) apoptotic pathway. To date, however, the detailed molecular mechanism of cell cycle arrest involved remains not fully understood.

Since most of patients with cholangiocarcinoma present at diagnoses with unresectable disease or the occurrence, in this cancer, of high-rate of post-resection relapse, the current adjuvant or palliative option is of limited benefit. It is mandatory, therefore, to evaluate new potential anticancer drugs. ZOL, due to its mechanism of action so far highlighted, could represent a reliable chemotherapeutic agent able to sensitize cells to cytotoxic agents and/or radiations.

Here we investigated, for the first time on two cholangiocarcinoma cell lines, the role of ZOL on cell morphology, proliferation, cycle phases, and apoptosis to regard its potential use in cholangiocarcinoma therapy.

2. Methods and materials

2.1. Reagents

Zoledronic acid (ZOL, ZOMETA[®], Novartis Europharm, UK) and gemcitabine (GEM, GEMZAR[®], Eli Lilly, Italy) were kindly provided by Drs. Vittorio Rizzoli and Maria Cristina Baroni, Dipartimento di Medicina Interna e Scienze Biomediche, Università degli Studi di Parma. ZOL was dissolved in phosphate buffer saline (PBS) as a 5 mM stock solution while GEM as 100 mM stock solution and both stored at –20 °C. RPMI 1640 medium, staurosporine, geranylgeraniol (GGOH), pamidronate, clodronate, and phosphorylated histone H3 (pHH3, 8656-R), were purchased from Sigma–Aldrich (St. Louis, MO, USA), and fetal bovine serum (FBS) from Gibco-BRL (Grand Island, NY, USA). Antibodies against Bcl-xL (H-5), Bax (P-19), TP53 (DO-1), Rap1A (SC-1482, unprenylated form) were obtained from Santa Cruz Biotechnology (Santa Cruz, Temecula, CA, USA). Monoclonal antibody against HSP27 (G3.1) was from StressGene (Victoria, BC, Canada); caspase-3, PARP, cyclin A, cyclin B1, cyclin D1, pRb1, Rb, and p27 antibodies were from Cell Signaling Technologies (Beverly, MA, USA); monoclonal anti-actin (AC-40) antibody was from Sigma–Aldrich. HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA) and the enhanced chemiluminescence system (ECL) were from Millipore (Millipore Co., Billerica, MA, USA). Reagents for electrophoresis and blotting analysis were obtained from BIO-RAD Laboratories (Richmond, CA, USA).

2.2. Cell culture

The cholangiocarcinoma cell lines EGI-1 and TFK-1 were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, GERMANY) and maintained in RPMI-1640 medium, containing antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and supplemented with 10% FCS and with 2 mmol/l glutamine. Cultures were kept in an incubator at 37 °C in a water-saturated 5% CO₂ atmosphere in air. Routine subcultivations were carried out every week. Cell morphology was assessed on May–Grünwald stained cells.

2.3. MTT assay

Viability of EGI-1 and TFK-1 cells was evaluated by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] (MTT) (Sigma–Aldrich) assay. 5000 cells/well were seeded into 96-well plates and left to adhere overnight. The cells were then incubated for 3 days in the absence or in the presence of 1–1000 µM ZOL. Then 100 µl of 1 mg/ml MTT was added for 1 h. After removal of the MTT, cells were treated with 100 µl DMSO and formazan product was solved. The absorbance was determined at 595 nm in a multiwell plate reader (BIO-RAD, Microplate Reader 555). Drug-induced proliferation changes were calculated as percentage of cell growth inhibition using the formula: $(1 - \text{extinction of treated sample} \times 100) / (\text{extinction of control sample})$. The half-maximal inhibition constant (IC₅₀) was determined using the nonlinear regression program CalcuSyn (Biosoft, Cambridge, UK).

2.4. Clonogenic assay

Colony formation was determined by seeding cells into a six-well culture plates at a density of 50 cells/cm² well and left to adhere overnight. The cells were then incubated in the absence or in the presence of 2–32 µM ZOL. After 10-day-incubation, the wells were washed twice with PBS, and fixed with 1% formaldehyde. They were then stained with 0.1% crystal violet. The colony is defined to consist of at least 40–50 cells. For the evaluation of cell growth, colonies were examined under a light microscope.

2.5. Western blotting

Total proteins were extracted by lysing the cells with a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.1% SDS and protease inhibitor cocktail followed by ultracentrifugation at 13,000 rpm for 10 min. Protein concentration was determined using a protein assay from BIO-RAD according to the manufacturer's protocol, using BSA as standard. Proteins (50 µg) were resolved by SDS/PAGE (12%) and blotted onto PVDF membranes (Millipore). Immunodetection was carried out in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, 5% non-fat dry milk, followed by HRP-conjugated secondary antibodies at 1:20,000 dilution. Detection was performed using an ECL kit.

2.6. Flow cytometric cell cycle and morphological analysis

Cholangiocarcinoma cells were incubated with 10 and 50 µM ZOL for 72 h. For the cell cycle assay EGI-1 and TFK-1 cells were harvested and subjected to centrifugation, 5×10^5 cells were incubated overnight at 4 °C in 1 ml of hypotonic fluorochrome solution. DNA content was determined by flow cytometry using a Coulter EPICS XL-MCL cytometer (Coulter Co., Miami, FL, USA) and the cell cycle phase distribution was analyzed by ModFit 2.0 software (Verity Software House, Topsham, ME, USA). Respective to their phase-specific DNA-content cells were divided into G1, S, G2/M phases and, when present, sub-G1 phase counted as apoptotic cells. Cell death was also assessed by morphological criteria on cells stained with Hoechst 33342 (a fluorescent cell-permeant DNA stain) and propidium iodide (PI, a fluorescent permeant stain for membrane damaged cells) using fluorescence microscopy.

3. Results

3.1. Growth inhibitory effects

In order to test the effect of ZOL on cholangiocarcinoma cell growth, EGI-1 and TFK-1 cell lines were treated with increasing

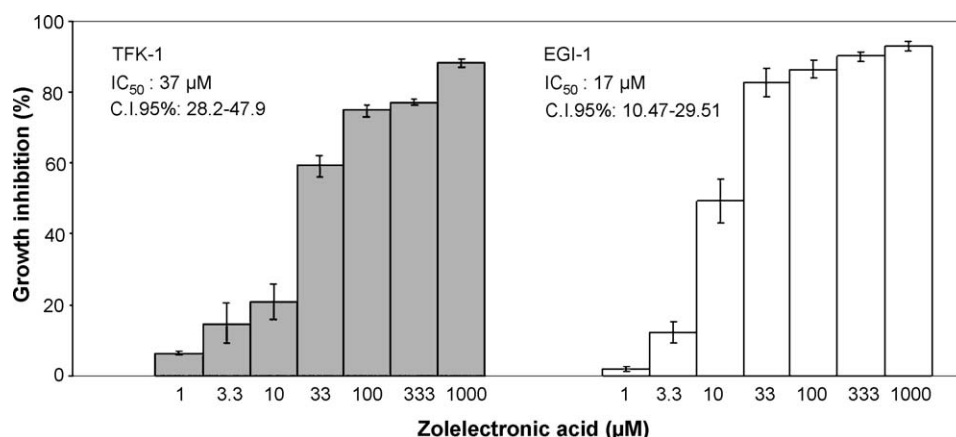


Fig. 1. ZOL inhibits EGI-1 (\square) and TFK-1 (\blacksquare) cell proliferation. Cells were exposed to ZOL at a concentration ranging from 1 to 1000 μ M. Following 72 h of incubation growth inhibition was analyzed by MTT assay. Data are presented as mean \pm S.D. of six determinations. A representative experiment is shown. IC_{50} values and confidential intervals are estimated from the data of three independent experiments.

concentration of ZOL (1–1000 μ M) for 72 h, followed by an MTT assay (see Section 2). As revealed in Fig. 1, after 72 h of ZOL treatment cholangiocarcinoma cells significantly reduced their growth in a dose-dependent manner. In fact, the tumor cell proliferation was reduced to about 21 and 50% for TFK-1 and EGI-1, respectively, at ZOL concentration of 10 μ M. Then inhibition increased to 59% in TFK-1 and 83% in EGI-1 at 33 μ M. Higher concentrations of drug further inhibited proliferation of both cell lines. The mean IC_{50} estimated from three independent experiments gave a concentration of 37 μ M (C.I.95%: 28.2–47.9) for TFK-1 cells and of 17 μ M (C.I.95%: 10.5–29.5 μ M) for EGI-1 cells.

3.2. Rescue by GGOH of morphological and proliferation changes induced by ZOL

Besides the significant inhibition of proliferation induced by ZOL at relatively high concentrations, concomitant alterations of cell morphology occurred. Control cultures (Fig. 2A) show typical morphological variations of cholangiocarcinoma cells from cuboidal-to-low-columnar epithelial cells with rounded to polygonal nuclei. Subsequent to 50 μ M ZOL treatment long and narrowed cells resembling fibroblasts coupled with a marked reduction of cell number are observed in EGI-1 cells. Similar changes, even if

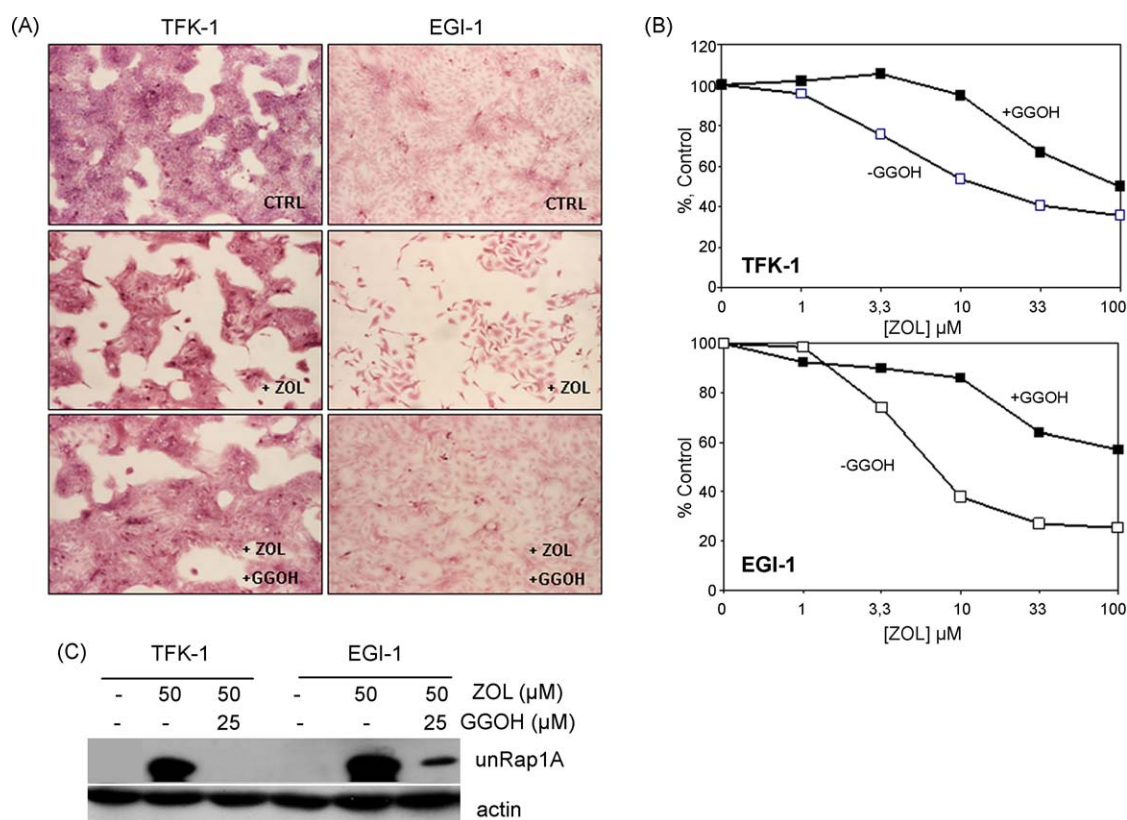


Fig. 2. Cholangiocarcinoma cell changes induced after ZOL treatment in the absence and in the presence of 25 μ M GGOH. (A) *Morphology*: EGI-1 cells were plated and then treated for 72 h with vehicle medium, 50 μ M ZOL, or both 25 μ M GGOH and 50 μ M ZOL. (B) *Proliferation*: cells were exposed to ZOL at a concentration ranging from 1 to 100 μ M in the presence (\blacksquare) and in the absence (\square) of 25 μ M GGOH. Following 72 h of incubation growth inhibition was analyzed by MTT assay. Data are presented as percentage of control. (C) *Western blotting*: loss of prenylation of Rap1A in EGI-1 and TFK-1 cell extracts after 72 h of 50 μ M ZOL treatment in the presence and in the absence of 25 μ M GGOH.

less marked were observed also in TFK-1 cells. To substantiate the mechanism of ZOL activity GGOH, an acyclic isoprenoid substrate of protein prenylation, was added during drug treatment. The addition of 25 μM GGOH determines a rescue of zoledronate-induced morphological changes in both cell lines.

The ability of GGOH to attenuate/rescue ZOL cytotoxicity was assessed by plating cells and then treating them with various ZOL concentrations or in combination with GGOH as described in the legend to Fig. 2B. By using Western blotting analysis in the presence of 50 μM of ZOL the band for the unprenylated form of Rap1A (a small GTP protein of Ras family) in both cell lines became visible, indicating high level of the unprenylated form. Co-treatment with GGOH significantly attenuated ZOL cytotoxicity, increasing the IC_{50} by 4.5- and 10-fold for TFK-1 and EGI-1 cells, respectively. Moreover, GGOH counteracted the ZOL-induced unprenylation of Rap1A in EGI-1 and TFK-1 cells (Fig. 2C). This ability of GGOH to alleviate/rescue ZOL toxicity could be ascribed to cell replenishing of substrates for protein prenylation.

3.3. Loss of clonogenic survival

Because of the pronounced cytostatic effects of ZOL on cell growth observed on monolayer cultures, we then verified if ZOL could affect the ability of EGI-1 and TFK-1 cells to form colonies *in vitro*. Clonogenic assay, based on the ability of a single cell to grow into a colony, is the method of choice to determine the effectiveness of cytotoxic agents. After 10 days in culture both EGI-1 and TFK-1 cells, seeded at 50 cells/ cm^2 , formed a large number of colonies clearly visible at naked eye (Fig. 3). However, when cells were grown for 10 days in the presence of 8 μM ZOL, number and size of TFK-1 and EGI-1 colonies were markedly reduced. When treated with 16 μM ZOL, both cell lines did not form visible colonies. These results clearly indicate that the ZOL treatment determines a long-lasting cell cycle arrest of cholangiocarcinoma cells suppressing their colony-forming ability in a dose-dependent manner.

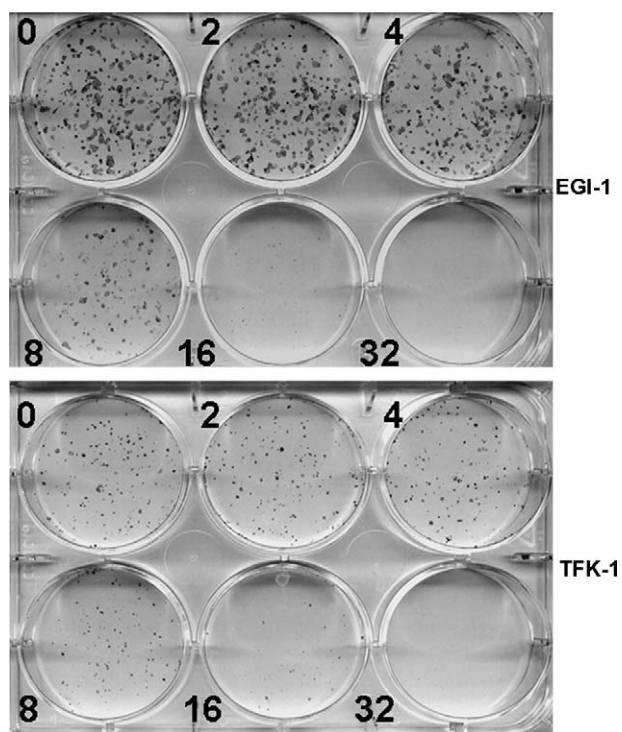


Fig. 3. Colony formation assay. Influence of 2–32 μM ZOL treatment for 10 days on the ability of EGI-1 and TFK-1 cells to form colonies. A representative experiment is shown. The experiment was performed three times yielding similar results.

3.4. Cell cycle phase distribution

We then investigated the effect of ZOL on the cell cycle phase distribution of cholangiocarcinoma cells following treatment with this drug over a 72 h period. The analysis (Fig. 4) revealed the cell cycle distribution of untreated control cells showing an average of 70 and 62% cells in G1, 9 and 11% in G2/M, and 21 and 28% in S-phase for EGI-1 and TFK-1, respectively. It is interesting to note that when EGI-1 cells were treated with 10 μM ZOL for 72 h, the proportion of cells in G1 was reduced to 34 and 55% of the cells occurred in S-phase, whereas only at 50 μM ZOL a marked accumulation of TFK-1 cells in S-phase (71%) occurred. As evident from this figure, the proportion of cells in the G2/M phase did not change significantly indicating no additional arrest in this phase. In EGI-1 cells a distinct loss of cell in G2/M phase at the highest drug concentration (50 μM) was detectable. Taken together, these results suggest that ZOL treatment for 72 h induced the entrapment of cholangiocarcinoma cells in the S-phase, resulting, therefore, in an effective reduction of cells capable to complete cell cycle, and thus leading to a diminished cell proliferation. Interestingly, in both cholangiocarcinoma cell lines, ZOL treatment did not induce any hypodiploid sub-G1 peak, a feature indicative of apoptotic cell death (Fig. 4, upper panel). It is worth to note that 48 h co-treatment with GGOH partially rescues the cell cycle phase distribution seen in ZOL-exposed cholangiocarcinoma cells (data not presented).

3.5. Expression of putative phase markers and cell cycle regulators

We then examined the mechanisms of ZOL-induced S-phase arrest by focusing on the expression level of phase-specific cyclins and the cell cycle regulators such as tumor suppressor pRb and p27^{KIP1}. Modified expression and/or phosphorylation of these molecules are indicative of which checkpoints may be involved. Checkpoints are important sensors that ensure the proper sequence of cell cycle events allowing cells to repair DNA damage. There are at least three checkpoints at G1/S, S and G2/M phases that can allow DNA repair and promote cell death in unrepaired cells. The first checkpoint at G1/S is often compromised in malignant cells due to deletion or loss of function of TP53, guardian of the genome, and/or mutation of pRb, main regulator of the cell cycle. As shown in Fig. 5, while we confirmed the absence of TP53 in TFK-1 cells as known from the literature data, its overexpression in EGI-1 cells is likely to be ineffective due to the presence of one deleterious transition mutation (G > A, R273H) in the DNA binding domain that prevents its binding to DNA [29]. pRb protein hinders the cell from replicating damaged DNA by preventing its progression along the cell cycle through G1 into S. In the hypophosphorylated state, pRb protein is active and carries out its role as tumor suppressor by inhibiting cell cycle progression. Phosphorylation inactivates pRb. As shown in Fig. 5, in both untreated cell lines the hyperphosphorylation status of pRb (SER 780) occurred and drug treatment did not affect the status of phosphorylation, except only in EGI-1 cells in the presence of 50 μM ZOL. The hyperphosphorylation status of pRb (SER 780) in both treated cell lines associated with a deletion (TFK-1) or deleterious mutation (EGI-1) of TP53 greatly suggests that the first checkpoint G1/S is compromised allowing unrestrained cells freely entering in the next S-phase.

We then examined the cyclins, putative markers of cell cycle phases and regulatory subunits of the cyclin-dependent kinases (cdks), whose levels peak the specific stages of the cell cycle. The first cyclin level examined was cyclin D1, which is maximally expressed in mid-to-late G1 phase and its kinase activity, elicited by binding with cdk4 (predominantly cyclin D1/cdk4 complex), is up-regulated in cancer cells [30]. As shown in Fig. 6, treating cells

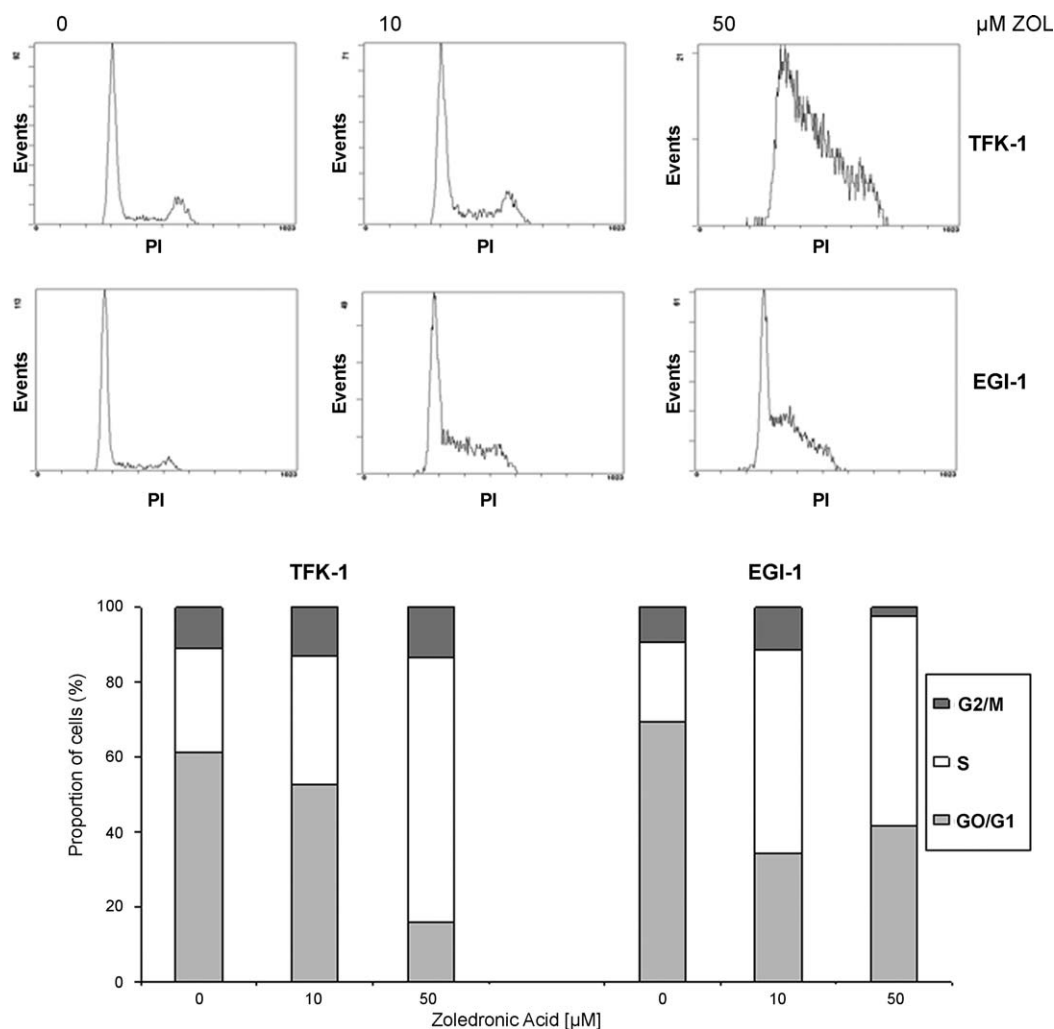


Fig. 4. Flow cytometric analysis of DNA content in EGI-1 and TFK-1 cells, in the absence or in the presence of 10 or 50 μM of ZOL for 72 h. The percentage of cells in each cycle phases, in a representative experiment as shown in barchart. Replicated experiments yielded similar results.

for up to 72 h with 10–100 μM ZOL resulted in a marked decrease of cyclin D1 in EGI-1 cells and only at higher concentrations in TFK-1. We further verified the status of p27^{KIP1} whose function is to bind to cyclin D either alone, or when complexed to its catalytic subunit cdk4. In this way, p27^{KIP1} inhibits the catalytic activity of cdk4, preventing cdk4 from adding phosphate residues to pRb protein. Increased levels of the p27^{KIP1} protein typically cause cells to arrest in the G1 phase of the cell cycle. Upon ZOL treatment a dose-dependent decrease in the level of p27 inhibitor was observed in both cell lines, more evident in EGI-1 cells. The p16^{INK4A}, which shares with pRb and p27 a regulatory role at G1/S checkpoint, is frequently mutated or deleted in a wide variety of tumors, in particular in bile duct cancers, and not expressed in the cholangiocarcinoma cell lines used in this work [31,32]. In several human malignancies, the loss, mutation or silencing of the gene encoding the CDK4 inhibitor p16^{INK4A} hyperactivates the kinase activity of CDK4 [33–35]. Next, cyclin A expression is important for progression through the S-phase and G2/M transition [36]. Cyclin A, in contrast to cyclin D1, showed significant increases in both cell lines following cell exposed to ZOL and thus indicating a slow-down and/or an arrest in the S-phase. Moreover, cyclin B1, in complex with cdk1, functions as an M-phase kinase [37]. No changes of cyclin B1 level were observed in both cell lines except a distinct drop at 50 μM ZOL in EGI-1 cells. Finally, we analyzed the phospho-histone H3, a marker of chromatin condensation. The Western blotting analysis indicated that ZOL increases the level of

this M-phase marker. This increment was in agreement with the increased level of cyclins A.

These findings on the significant changes in the levels of specific cell cycle cyclins and inhibitors confirm the results observed with cell cycle analysis and further indicate a ZOL interference with the cell cycle progression machinery at the putative S-phase checkpoint.

3.6. Apoptosis assessment

As shown in Fig. 4, in both cholangiocarcinoma cell lines, ZOL treatment did not induce any apoptotic sub-G1 peak. In order to confirm the absence of apoptotic death, we examined the morphological and biochemical features of apoptosis. The cells were stained with Hoechst 33342 and propidium iodide. Condensed chromatin or apoptotic cells were rarely seen in all the cell populations studied: only 8–12% of control cells and of tested cells treated with the drug up to 25 μM were homogeneously and intensively stained by propidium iodide indicating cell death. No evident characteristics of apoptosis (such as cell shrinkage, apoptotic bodies, cytoplasmic blebs, chromatin collapse into patches and dense spheres) were observed. Only when EGI-1 cells were treated with 50 μM, approximately 10% of cells showed some apoptotic characteristics (not shown).

We then investigated the occurrence of apoptosis by biochemical criteria such as activation of caspases or the presence of

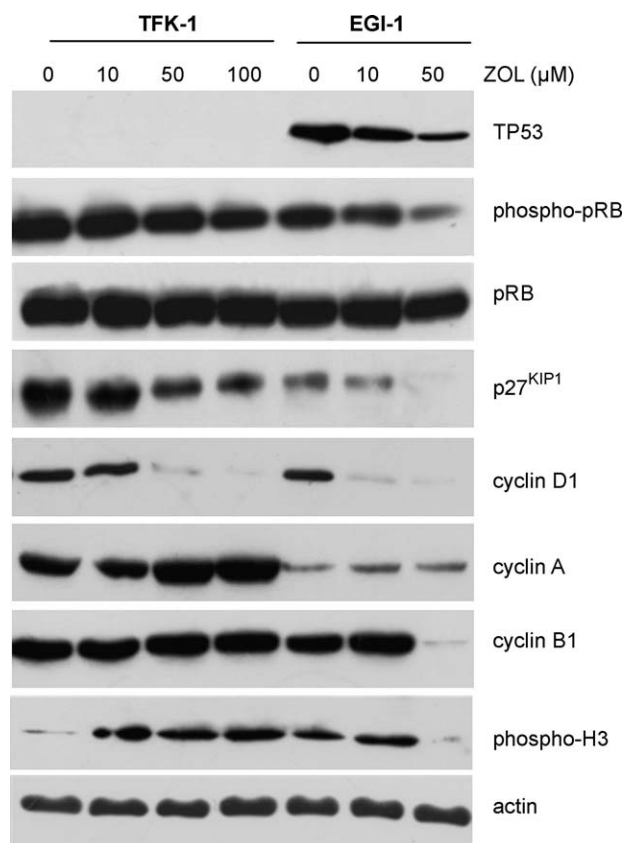


Fig. 5. Cell cycle regulators and inhibitors. Western blotting detection of TP53, pRb, phospho-pRb, p27^{KIP1}, cyclin D1, cyclin A, cyclin B1, phospho-HH3 in EGI-1 and TFK-1 cells after 72 h ZOL treatment. A representative experiment is shown.

cleavage products of their substrates. Fig. 6A shows that no effect on caspase-3 activation occurred after cells exposure to ZOL. Moreover, the drug did not induce cleavage of PARP, a key enzyme in the apoptotic cascade and substrate of active caspase-3.

To investigate if the cell-death resistance is specific to zoledronic acid, we evaluate the effect of staurosporine, gemcitabine and nitrogen (pamidronate) and a non-nitrogen (clodronate) containing bisphosphonate on caspase-3 and PARP activation. Experiments using the abovementioned drugs indicated that staurosporine on EGI-1 cells and gemcitabine on both cell lines were able to induce caspase-3 and PARP activation. In contrast and similar to ZOL, pamidronate and clodronate did not activate caspase-3 nor PARP (Fig. 6B).

The analysis of the expression of anti- versus pro-apoptotic proteins indicated that untreated TFK-1 cells markedly express the anti-apoptotic Bcl-xL and at greater extent the pro-apoptotic Bax protein, according to the literature data [38,39]. As shown in Fig. 6C, in TFK-1 cells ZOL treatment significantly affected the balance of anti- versus pro-apoptotic proteins; it is also interesting to note the marked decrease of Bax coupled to the increasing concentrations of ZOL. Moreover, in EGI-1 cells ZOL treatment did not affect the ratio of Bcl-xL/Bax, already shifted versus the anti-apoptotic Bcl-xL protein.

We then addressed the question whether the observed apoptosis resistance of S-phase arrested cells was associated to high expression of HSP27. HSP27, a powerful chaperone, is a stress protein induced in response to a variety of insults including oncogenic transformation and chemotherapeutic agents. Its expression allows/helps tumor cells to survive otherwise lethal conditions by counteracting at multiple control points of the apoptotic pathway and therefore increasing the tumorigenic potential of cancer cells [40]. As shown in Fig. 6C, constitutive expression of HSP27 was clearly evident in untreated TFK-1 cells; however, ZOL treatment did not affect its expression. In contrast, in EGI-1 cells HSP27 was not present and ZOL treatment did not induce its expression.

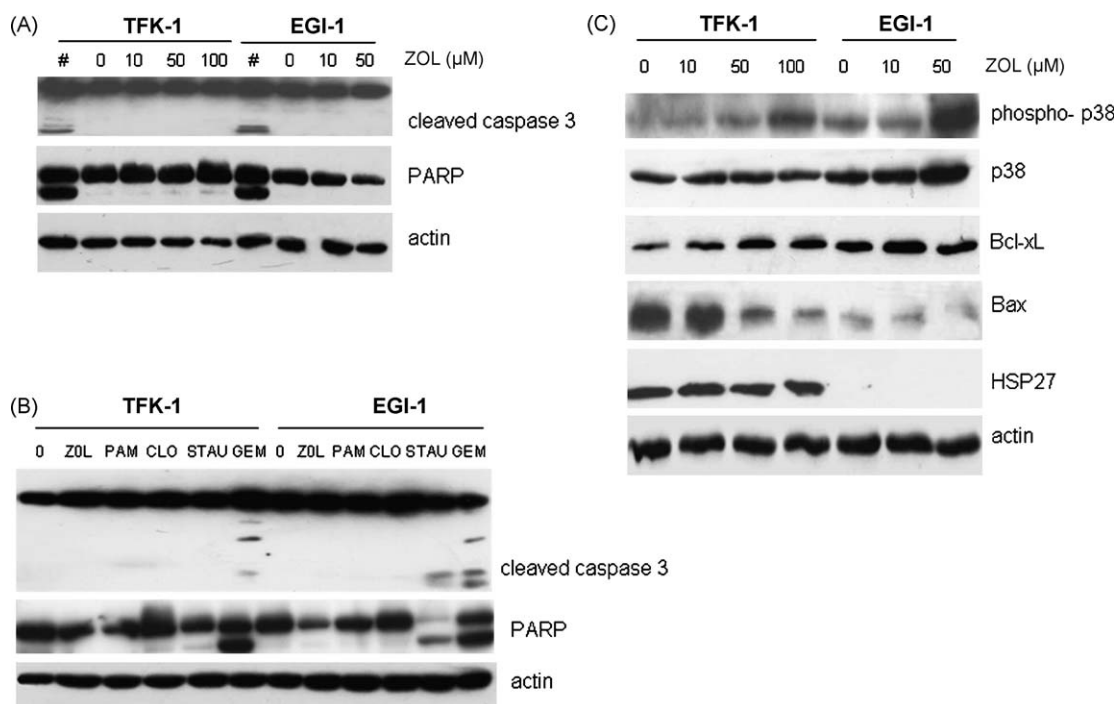


Fig. 6. Markers of apoptotic pathways. (A) Western blotting detection of caspase-3, PARP after 72 h ZOL treatment. The sign (#) indicates the positive control (TFK-1 and EGI-1 cells treated for 72 h with 100 μM of gemcitabine, a known apoptotic inducer). (B) Western blotting detection of caspase-3, PARP after 50 μM ZOL, 50 μM pamidronate (PAM), 500 μM clodronate (CLO), 500 nM staurosporine (STAU) and 100 μM gemcitabine (GEM) treatment for 72 h. (C) p38 protein, Phospho-p38 Bcl-xL, Bax, HSP27 in EGI-1 and TFK-1 cells after 72 h ZOL treatment. A representative experiment is shown.

4. Discussion

Despite recent improvements in surgery and the development of different regimens of multidrug chemotherapy over the past 25 years, survival of patients suffering from cholangiocarcinoma remains around 5–20% after 5 years [41,42]. The poor prognosis of cholangiocarcinoma [2] warrants new therapeutic strategies to improve the overall rate of survival, especially in high-risk subgroups.

In the present study, we examined the commonly used biphosphonate molecule ZOL, because of its high biological activity in inducing inhibition of proliferation and apoptosis in several cancer cell lines [20]. We here reported that ZOL, the most potent nitrogen-containing BP, causes antiproliferative effects in EGI-1 and TFK-1 cholangiocarcinoma cells. ZOL inhibited cell growth in dose-dependent manner in both cell lines tested. The IC_{50} range from 17 to 37 μ M for EGI-1 and TFK-1 cells, respectively. The co-treatment with GGOH prevented morphological changes and growth inhibition induced by zoledronate suggesting that a replenishing of protein geranylation could overcome the FPP synthase-block. Furthermore, ZOL-induced inhibition of Rap1A prenylation was reversed by GGOH, suggesting that ZOL-induced inhibition of proliferation is mediated via inhibition of the mevalonate pathway also in cholangiocarcinoma cells.

The ability to form a colony from a single cell, may be seen as a surrogate of the invasive ability of the tumor cell [43]. Interestingly, in cholangiocarcinoma cells this ability is markedly impaired at 8 μ M ZOL, a concentration 2–4-fold lower than those seen in the MTT assay. It should be noted that cells, exposed up to IC_{50} ZOL for 72 h, following drug removal later on resume their growth thus indicating the reversibility of the inhibition. In our opinion, this property of ZOL suggests its potential use, in cholangiocarcinoma affected patients, as effective adjuvant therapy for metastasis and disease relapse.

The antiproliferative effect of ZOL was associated with a change of cell distribution in the cell cycle phases as indicated by the accumulation and arrest of cancer cells in S-phase. This observation is in accordance with the previous studies on different types of tumor cells, all suggesting arrest of ZOL-treated cells in the S-phase [23–27]. To date, however, the details of the molecular mechanism of cell cycle arrest involved remain obscure. The G1/S checkpoint is often compromised in many tumor cells due to mutation or deletion of tumor suppressor genes including pRb and TP53 [44]. Several studies conducted so far have assessed the deregulation of the pRb-pathway components in various human tumors and cell lines, provided these pathway alterations play an obligatory role in tumorigenesis. The concomitant high level of p27^{KIP1} is required for the assembly of active cyclin D1-CDK4 [45]. In the presence of ZOL in both cholangiocarcinoma cell lines a reduced expression of the inhibitor p27^{KIP1} and a concomitant suppression of the cell cycle promoter D1 were observed. The dual suppression of cyclin D1 and p27^{KIP1}, two critical control molecules, and the persistence of pRb in the hyperphosphorylated status should allow the cells to freely enter in the S-phase and to accelerate through the cell cycle [45]. The dose-dependent accumulation of cells in the S-phase, however, suggests an arrest at the S-phase checkpoint. Moreover, the observed ZOL dose-dependent increase of cyclin A expression is consistent with a large fraction of cells accumulated in the S-phase. Massive DNA repair should presumably allow slow down or arrest of S-phase progression. The concomitant high level of cyclin B1, putative marker of M phase, was not significantly affected by ZOL, thus indicating that this phase has not been passed by the cells.

Taken together our results on phase distribution of cells, expression of cyclin proteins and their regulators pRb and p27^{KIP1} suggest that ZOL treatment on cholangiocarcinoma cells do not

induce G1/S arrest, as already seen in other tumor cells [26,46]. It determines an arrest of cells in the S-phase without an accumulation of cells in the G2-phase and absence of apoptosis induction.

Several studies have described induction of apoptosis (caspase-dependent and -independent) by the administration of ZOL to cancer cells. For example, in breast cancer ZOL decreased the cell growth in a dose-dependent manner, this effect being ascribed to the concomitant BP-induced increase in cell apoptosis [47]. Similarly, it was reported that ZOL was able to induce apoptotic death in several pancreatic cells achieving 35–90% of apoptotic cells in the treated groups. Independent of the TP53 and pRb status, in osteosarcoma cells, it was recently described a ZOL induction of atypical apoptosis independent of caspase activation and involving the mitochondrial pathway, in particular the translocation to the nucleus of the apoptosis-inducing factor (AIF) [27]. Our data support the notion that ZOL could inhibit cholangiocarcinoma cell proliferation by inducing cell accumulation/arrest in the S-phase but without inducing apoptosis. We have found that, subsequent to ZOL treatment both TFK-1 and EGI-1 cells are prevented to activate the apoptotic pathway as shown by the lack of sub-G1 peak, and the absence of caspase 3 cleaved fragment. This outcome was confirmed by the lack of morphological features of apoptosis.

This lack of apoptosis was not specific to ZOL treatment since similar results were obtained by using pamidronate (N-BP) or clodronate (BP). It is worthy to note that these cells are quite resistant to cell death as shown by moderate caspase-3 activation observed in the presence of a high concentration of staurosporine (a classical inducer of apoptosis in several cells). Interestingly, in staurosporine-exposed PC-3 cells the overexpression of anti-apoptotic protein Bcl-xL was found to be associated with resistance because of abrogation of cytochrome c release to the cytosol [48].

Discrepant observations about the ability of biphosphonates to induce apoptosis were reported by Lee et al. [49]. These authors showed that reduction in prostate cell growth was mainly mediated by cell cycle prolongation. Other two papers related to breast and lung cancer [50,51] showed that ZOL administration inhibited cancer cell proliferation, although only a minor fraction of cells (less than 20% in the case of lung cancer) entered in the apoptotic process.

Some mechanisms have been proposed to explain the potential refractory of tumor cells to activate the apoptotic program following ZOL treatment. For instance, the failure of ZOL to induce apoptosis is linked to the high levels of anti-apoptotic signals such as Bcl-2 proteins family. Interestingly, some authors have hypothesized that high concentrations of anti-apoptotic proteins, such as Bcl-2 proteins, within the biliary tree could play a key role in the development of cholangiocarcinoma [38,39]. A few years ago it was reported that the enhanced Bcl-2 expression alters the threshold for apoptosis in a cholangiocarcinoma cell line [52]. Moreover, Bcl-2 overexpression could protect myeloma cells against ZOL-induced apoptosis but not against cytostasis [53]. In agreement with this report, our results show that Bcl-xL, an anti-apoptotic member of the Bcl-2 family proteins, is significantly expressed in both cholangiocarcinoma cell lines, while the pro-apoptotic Bax protein is largely expressed only in TFK-1 cells. ZOL treatment reverted the ratio between pro-apoptotic Bax versus anti-apoptotic Bcl-xL in TFK-1 cells, meanwhile in EGI-1 cells this ratio, already shifted versus the anti-apoptotic component, was unaffected by the drug.

Our results support the plausible anti-apoptotic role of the Bcl-xL protein to contrast the cytotoxic effect of ZOL as observed by other authors [38]. As already indicated above, Bcl-xL overexpression is one of the mediator of resistance to staurosporine-induced apoptosis [48]. Recently, it was shown that Bcl-xL overexpression protects from apoptosis induced by HMG-CoA

reductase inhibitors (simvastatin) in murine tubular cell line [54]. Moreover, antisense Bcl-xL down-regulation has been reported to induce apoptosis in colorectal cancer cells [55]. Taken together, these reports and our results suggest that the expression of Bcl-xL protein might prevent cellular apoptosis, and confirm, at least *in vitro*, the resistance of TFK-1 and EGI-1 cells to the apoptosis-inducing effects of ZOL. An additional mechanism that emerges from this study and might explain the higher resistance to ZOL exhibited by TFK-1 cells, in comparison to EGI-1 cells ($IC_{50} = 37$ vs 17), could be linked to the constitutive expression of the anti-apoptotic HSP27 protein. This remark is suggested by the known role of HSP27 in conferring chemotherapy resistance, in inhibiting apoptotic cell death, and facilitating tumor progression [56,57]. Our previous results suggest that HSP27 could be also a prognostic factor in cholangiocarcinoma [58].

In conclusion, our study provides the first evidence that ZOL elicits a direct inhibitory action on the proliferation of cholangiocarcinoma cells. The cell cycle alteration induced by ZOL, i.e. cell accumulation/arrest in S-phase, was confirmed by changes in the level of cyclins D1 and A, and of regulators p27^{KIP1} and pRb. ZOL therefore, could represent a reliable chemotherapeutic agent able to sensitize cells to cytotoxic agents and/or radiations.

Our data indicate that a dose of 10–50 μ M ZOL is required for a significant cell cycle arrest in cholangiocarcinoma cells. This range of concentrations, however, may preclude ZOL to be used as monotherapy in cholangiocarcinoma, thus making mandatory the need to investigate differently active molecules to associate with ZOL, in order to obtain a synergistic therapeutic improvement and minimizing, in the same time, the side effects.

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