

Cytotoxic effects of a novel pyrazolopyrimidine derivative entrapped in liposomes in anaplastic thyroid cancer cells *in vitro* and in xenograft tumors *in vivo*

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Abstract

In this study, we evaluated the activity of two novel pyrazolopyrimidine derivatives (Si 34 and Si 35) against ARO cells, a human anaplastic thyroid cancer cell line. ARO cells exposed to different concentrations of the drugs showed a reduced growth rate and an increase of mortality. After 72 h incubation, doses of 5 and 10 μ M Si 34 determined a decrease of cell counts by \sim 25% and \sim 75% compared with those of control cells respectively. Similar findings were observed using Si 35. Treatment with both Si 34 and Si 35 at 10 μ M increased cell mortality also (\sim 29% and \sim 18% respectively). At these concentrations, a decrease in cyclin D1 levels was observed. To improve the biopharmaceutical properties, a liposome formulation was prepared. When entrapped in unilamellar liposomes, Si 34 exerted its cytotoxic effects even at lower doses (maximal inhibition at 5 μ M) and after shorter incubation time (48 h) either in ARO or other thyroid cancer cell lines. The effects were associated with weak apoptotic death. Inhibition of epidermal growth factor-stimulated src and ERK phosphorylation, as well as reduction of migration properties of ARO cells was also observed. Moreover, the growth of tumor xenografts induced in severe combined immunodeficiency (SCID) mice was inhibited by i.v. administration of 25–50 mg/kg of the drug liposomal formulation. In conclusion, the liposomal preparation of this novel pyrazolopyrimidine derivative appears to be a promising tool for the treatment of anaplastic thyroid cancer.

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Introduction

The outcome of anaplastic thyroid carcinoma is far from satisfactory. Most cases are subjected to multimodal treatment regimens based on aggressive surgery, external beam radiation and chemotherapy, but this approach is rarely effective, and the prognosis for these patients is still poor (Ain 1998, Pasieka 2003). However, promising results are being obtained in some patients thanks to combined therapies using novel molecular targeting agents (Kundra & Burman 2007).

Targeted molecular therapy is rapidly emerging as a promising strategy for the treatment of cancer. Molecular characterization of the development and progression of specific types of cancer (Gibbs 2000) has identified various classes of molecules that might be productively targeted by novel anticancer agents. One of the most promising is the protein tyrosine kinase (PTK) family. These enzymes participate in various signaling pathways that control cell proliferation, secretion, adhesion, and responses to mitogen

stress (Erpel & Courtneidge 1995, Parsons & Parsons 1997). PTK malfunction is a hallmark of many types of human neoplastic disease. Indeed, PTKs are encoded by the vast majority of oncogenes and proto-oncogenes that have been implicated in human cancer. Regulation of thyrocyte growth also involves activation of PTKs, including growth factor receptors and elements of signal transduction pathways whose deregulation is thought to lead to their neoplastic transformation (Fagin 2004). Of the various PTK inhibitors being tested as potential anticancer agents (Zwick et al. 2001), those that suppress the activity of non-receptor PTKs of the Src family have attracted particular interest (Alvarez et al. 2006). Several pyrazolo[2,3-d]-pyrimidines have been identified as potent and selective inhibitors of c-src activity. Two of these, the pyrazolopyrimidine derivatives PP1 and PP2, have also been shown to block RET-induced thyroid tumorigenesis (Carlomagno et al. 2002, 2003).

In previous studies, a series of novel compounds that are structurally similar to PP1 and PP2 has shown inhibition of epidermal growth factor (EGF)-stimulated src activation resulting in arrest of proliferation of A431 epidermoid tumor cells. In particular, two of these compounds, Si 34 and Si 35, exerted a strong antiproliferative effect also against a breast cancer cell line (Carraro et al. 2004, Schenone et al. 2004a,b).

The aim of the present study was to investigate the effects of Si 34 and Si 35 against ARO cells, a well-established undifferentiated thyroid cancer cell line widely used to test *in vitro* the effects of novel potential anticancer drugs (Fagin et al. 1993). Moreover, a liposomal drug delivery system was prepared to improve the biopharmaceutical features of the pyrazolopyrimidine derivatives, such as the possibility to achieve a stable aqueous drug dispersion thus avoiding the toxic effect of any organic solvent, e.g., dimethylsulfoxide (DMSO), to be used to solubilize lipophilic drugs. The liposomal preparation of Si 34 was also tested in other thyroid cancer cell lines. Moreover, its effects were investigated on cell proliferation signal transduction pathways, apoptosis and invasiveness of ARO cells. Finally, the effect on the growth of ARO cells in non obese diabetic/severe combined immunodeficiency (NOD-SCID) mice was analyzed.

Methods

Compounds

N-benzyl-1-(2-chloro-2-phenylethyl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (Si 34) and 1-(2-chloro-2-phenylethyl)-6-(methylthio)-*N*-(2-

phenylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (Si 35; Fig. 1) were synthesized as described (Carraro et al. 2004, Schenone et al. 2004b). Briefly, ethyl 5-amino-1-(2-hydroxy-2-phenylethyl)-1H-pyrazolo-4-carboxylate was reacted with benzoyl isothionate and subsequently cyclized with sodium hydroxide. The resulting pyrazolopyrimidine derivative was then chlorinated and reacted with amines. The pyrazolopyrimidine structure of Si 34 and Si 35 is identical to that of PP1 and PP2, but there is a thiomethyl group at C6, a 2-chloro-2-phenylethyl chain at N1, and different amino groups at C4. Stock solutions of each compound were prepared in 100% DMSO (50 mM/l) and diluted with culture medium before addition to cell cultures.

Molecular modeling

The molecular modeling work was carried out coupling two different computational methods (conformational analysis and ClogP estimation) as reported

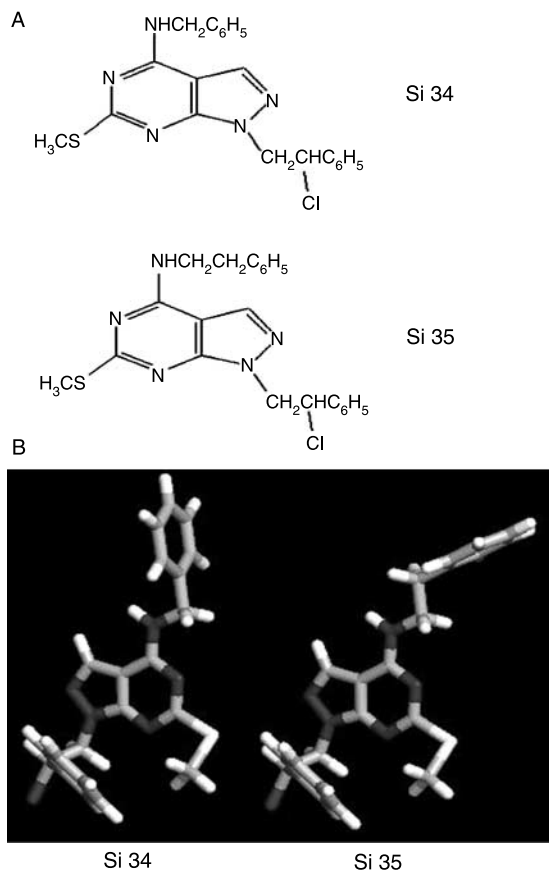


Figure 1 Structure of the two pyrazolopyrimidine derivatives used in this study. (A) Chemical structures of compounds Si 34 and Si 35. (B) Global minimum energy conformations of compounds Si 34 and Si 35.

in a recent communication (Alcaro *et al.* 2007). The structures of Si 34 and Si 35 were built using the Maestro Graphical interface and modeled using the Merck Molecular Force Field (MMFF) in Gibbs Born/Surface Area (GB/SA) water as implemented in MacroModel ver 7.2 (Mohamadi *et al.* 1990). For each compound, 1000 conformations were generated by Monte-Carlo search and de-duplicated using standard conditions. The lipophilicity of both compounds was estimated by the ClogP method (Leo & Hansch 1971) using the SMILE notation format as input of the web interface.

Preparation of Si 34-loaded liposomes

Liposomes were made up of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate/cholesterol/*N*-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (all purchased by Genzyme, Suffolk, UK) in a 6:3:1 molar ratio. Si 34-loaded liposomes were prepared by dissolving lipid components into a pyrex round-bottomed flask using a solvent mixture of chloroform/methanol (3:1 v/v; Sigma-Aldrich S.r.l). Then, a solution (200 µl) of the lipophilic drug (1 mg/ml) in chloroform/dichloromethane was added to the solvent mixture previously prepared with the aim to encapsulate the drug into liposomal bilayers. Organic solvents were removed by means of a rotavapor Büchi 461 under a slow nitrogen flux, thus achieving the formation of a thin film on the inner walls of the glass tube. The lipid film was hydrated with 1 ml of a saline solution (0.9% w/v NaCl) and submitted to three alternate cycles (3 min each) of warming to 58 °C in a thermostated water bath and vigorous mixing by vortex at 11 × *g*. The obtained multilamellar liposomes were kept at 57–60 °C for 3 h to anneal the bilayer structure. To reduce both liposome lamellarity and mean size, multilamellar vesicles were extruded at 60 °C by Lipex Extruder (Vancouver, Canada) through polycarbonate membrane filters (Costar, Corning Incorporated, New York, NY, USA). At first, the liposomes were extruded by two polycarbonate filters of 400 nm pore size (10 cycles) and then by two polycarbonate filters of 200 nm pore size (10 cycles). The working pressure was 450 kPa for 400 nm and 880 kPa for 200 nm pore-size filters. The extrusion procedure led to the formation of unilamellar liposomes. Si 34-loaded unilamellar liposomes were purified from eventually unloaded drug by gel permeation chromatography as previously reported (Calvagno *et al.* 2007). The amount of Si 34 added during liposome preparation that became entrapped was ~98%.

Cell cultures and cell growth analysis

The ARO and FRO cells derived from a human anaplastic thyroid carcinoma, BCPAP, TPC-1, and BHP 17-10 derived from poorly differentiated papillary thyroid carcinoma, and FTC-133 cells derived from a follicular thyroid cancer were grown as previously described (Hoelting *et al.* 1995, Ohta *et al.* 1996, Russo *et al.* 2001). Control cells were exposed to DMSO alone at the same concentration present in cultures exposed to the test compounds (i.e., 1:5000 – 0.02% when final concentrations of 10 µM/l compounds were used).

For cell growth rate analysis, 10⁴ cells were plated in 60 mm dishes. Twelve hours later, when the cells had presumably settled and begun to proliferate, test compounds were added. Cells were harvested after 24, 48, and 72 h incubation and counted in a Neubauer chamber. The growth was also evaluated using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and measure of ³H-thymidine incorporation. For MTT assay, 3000 cells were seeded onto 96 well plates in 100 µl medium. Twenty-four hours later, medium was changed, test compounds (1, 5, and 10 µM/l Si 34 free or entrapped in liposomes or 1, 5, and 10 µM/l Si 35) or equivalent dilutions of DMSO (control) were added, and plates were incubated for 24, 48, and 72 h. The solubilized formazan product was spectrophotometrically quantified with a microplate spectrophotometer (Multiskan MS 6.0, Labsystems, Helsinki, Finland) at a wavelength of 540 nm with reference at a wavelength of 690 nm. ³H-thymidine incorporation experiments were performed as described previously (Damante *et al.* 1990).

Cell viability was evaluated by trypan blue dye exclusion assay, as previously described (Navarra *et al.* 2001). Briefly, cells exposed to test compounds (see above) were trypsinized, the pellet was re-suspended in 0.4% trypan blue buffer, and cells were counted in a Neubauer chamber. Cell death rates were expressed as the percentage of stained cells over total cells that were counted.

Protein extraction and western blot analysis

Total proteins were extracted from thyroid cell lines as previously described (Russo *et al.* 2005). Briefly, confluent cells from three Petri dishes were collected and homogenized in 1 ml buffer containing Tris-HCl 50 mM, NaCl 150 mM, Triton 1%, sodium deoxycholate 0.25%, sodium pyrophosphate 10 mM, NaF 1 mM, sodium orthovanadate 1 mM, phenylmethylsulphonyl fluoride 2 mM, leupeptin 10 µg/ml, and aprotinin 10 µg/ml (all from Sigma-Aldrich S.r.l). The homogenate was centrifuged at 10 000 *g* (4 °C for 10 min),

and the supernatant containing the whole-cell lysate was quantified spectrophotometrically using the Bradford method. Twenty micrograms of proteins were loaded onto a 7.5% SDS–polyacrylamide gel and subjected to electrophoresis at a constant voltage (120 V). Electroblothing to a Hybond ECL–PVDF nitrocellulose membrane (Amersham Pharmacia Biotech.) was performed for 2 h at 125 mA using the Mini Trans Blot system (Bio-Rad Laboratories S.r.l.). Membranes were blocked with Tween/Tris buffered saline (TTBS)/milk (TBS, 1% Tween 20, and 5% non-fat dry milk) for 2 h at room temperature and incubated with an affinity-purified anti-poly ADP-ribose polymerase (PARP) monoclonal antibody diluted 1:2000 (Sigma–Aldrich), or a 1:500 dilution of anti-cyclin D1 antibody, anti-ERK2 and anti-p-ERK (1:1000 and 1:100 dilution respectively (Santa Cruz Biotech., Santa Cruz, CA, USA) or anti-src and anti-p-src (1:500 dilution; Cell Signaling Technology, Danvers, MA, USA). For normalization purposes, immunoblots were incubated overnight at 4 °C with mouse monoclonal anti-human β -actin antibody (Sigma–Aldrich S.r.l.) diluted 1:5000 in TTBS/milk. After one 15 min and two 5 min washes in TTBS, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse antibody (Transduction Laboratories, Lexington, KY, USA) diluted 1:10 000 in TTBS/milk. After one 15 min and two 5 min washes in TTBS, the protein was visualized with an enhanced chemiluminescence western blot detection system (ECL plus, Amersham Pharmacia Biotech).

Analysis of nuclei morphology by fluorescence microscopy

Cells were plated on glass cover slips and incubated with the tyrosine kinase inhibitors or with paclitaxel (Sigma–Aldrich). After two washes with PBS, cells were fixed for 20 min at 37 °C with 4% paraformaldehyde. Then, cells were washed with PBS and stained with 1 μ g/ml Hoechst 33258 (Bio-Rad Laboratories) for 20 min at 37 °C in the darkness. After two PBS washes, the cells were mounted on slides with Slow-Fade reagent (Molecular Probes, Leiden, The Netherlands) and observed with a Nikon Diaphot fluorescence microscopy at an excitation wavelength of 365 nm.

Annexin V staining

Cells were treated with the appropriate drugs or 2 μ M/l doxorubicin (Sigma–Aldrich) as positive control. After 48 h incubation cells were detached by trypsin, washed twice with cold PBS, and then suspended in 1×10^6 cells/ml.

Hundred microliters of the suspension were transferred to a 5 ml culture tube and 5 μ l FITC Annexin V (BD Biosciences, San Jose, CA, USA) were added. The samples were gently vortexed and incubated for 15 min at 25 °C in the darkness. Finally, 400 μ l of $1 \times$ Binding Buffer were added to each tube and the samples were analyzed by flow cytometry within 1 h. The used flow cytometer was a FACSCalibur (BD Biosciences) and the analysis was performed with Cell Quest software (Becton Dickinson European HQ, Erem bodegem–Aalst, Belgium).

Analysis of invasiveness

Chemoinvasion experiments were performed with the Matrigel Invasion Chambers constituted by 24 well plates equipped with 8 μ m pore size polycarbonate filters overcoated with matrigel (Corning Inc., New York, NY, USA). ARO cells were seeded in the upper compartment (1×10^4 cells/well) and treated with the liposomal Si 34 formulation and with the free drug at the concentrations of 5 and 10 μ M/l and then incubated at 37 °C for 24 and 48 h. EGF (20 ng/ml) was used as the chemoattractive molecule in the lower compartment of the chamber. After the incubation periods, the upper surface of the filter was wiped off, and the remaining cells that traversed the Matrigel and spread on the lower surface of the filter were collected separately and an MTT assay was then performed. The percentage of cell viability of each chamber was calculated with respect to the control (untreated cells).

In vivo experiments

The animal experiments were conducted in accordance with the principles and procedures outlined by the local Ethical Committee.

ARO cells (1×10^6) were diluted in 100 μ l PBS and injected s.c. on the flank of immunodeficient NOD-SCID mice. When the tumor reached a 10 mm diameter, two groups of mice ($n=5$ each) were treated intravenously with a daily dose of 300 μ l physiologic solution containing 25 and 50 mg per kilogram of Si 34 in the liposomal formulation. Control mice ($n=5$) received 300 μ l physiologic solution. The sizes of the tumors were measured at regular intervals with a caliper, and tumor volumes calculated according to the formula: length \times width \times height $\times 0.5236$. The body weight, feeding behavior, and motor activity were used as indicators of general health.

Statistical analysis

All data were expressed as means \pm s.e.m. Differences between the various experimental points were evaluated with one-way ANOVA followed by the Turkey-Kramer multiple comparisons test using GrafPAD Software for Science (San Diego, CA, USA).

Results

Structural features of the novel pyrazolopyrimidine derivatives

We have previously demonstrated that the antitumoral effects of a series of novel pyrazolopyrimidine derivatives endowed with tyrosine kinase-inhibiting activity (Carraro *et al.* 2004, Schenone *et al.* 2004b, Angelucci *et al.* 2006). In the present study, we tested the effects of two of these compounds, Si 34 and Si 35 (Fig. 1), on the growth rate and the viability of anaplastic thyroid cancer cells. The computational analysis revealed a different conformational space for the two compounds, whereas their most stable structures were very similar (Fig. 1B). In fact, the methylene difference that distinguishes Si 35 from Si 34 seemed to have no significant effect on the other flexible moieties of the common scaffold. Both compounds were highly lipophilic, reflecting a high capacity for diffusion across cell membranes, with limited water solubility.

Effects on the growth and mortality of ARO cells

Seventy-two hours of exposure to 10 μ M Si 34 or Si 35 significantly inhibited the growth of ARO cells (Fig. 2). After exposure to 5 and 10 μ M Si 34, ARO cell counts were decreased by \sim 25% and 75% respectively, compared with those of control cells treated with corresponding dilutions of DMSO. Similar reductions were observed with 5 and 10 μ M Si 35 (-35% and -70% respectively; Fig. 2). At 25 μ M, both Si 34 and Si 35 killed almost all the cells while no significant effect on cell growth was observed with lower concentrations of the compounds or shorter incubation times (data not shown). Similar results emerged when the compounds' effects on cell growth were assessed with the MTT assay (Fig. 2) and 3 H-thymidine incorporation assay (data not shown). The tyrosine-kinase inhibitors also increased cell mortality. In trypan blue exclusion assays (Fig. 2), ARO cell mortality rates were roughly four and seven times higher than controls after 72 h incubation with 5 and 10 μ M Si 35, and more substantial increases (4- and 12-fold) were observed after exposure to Si 34. Examination of Cyclin D1 levels

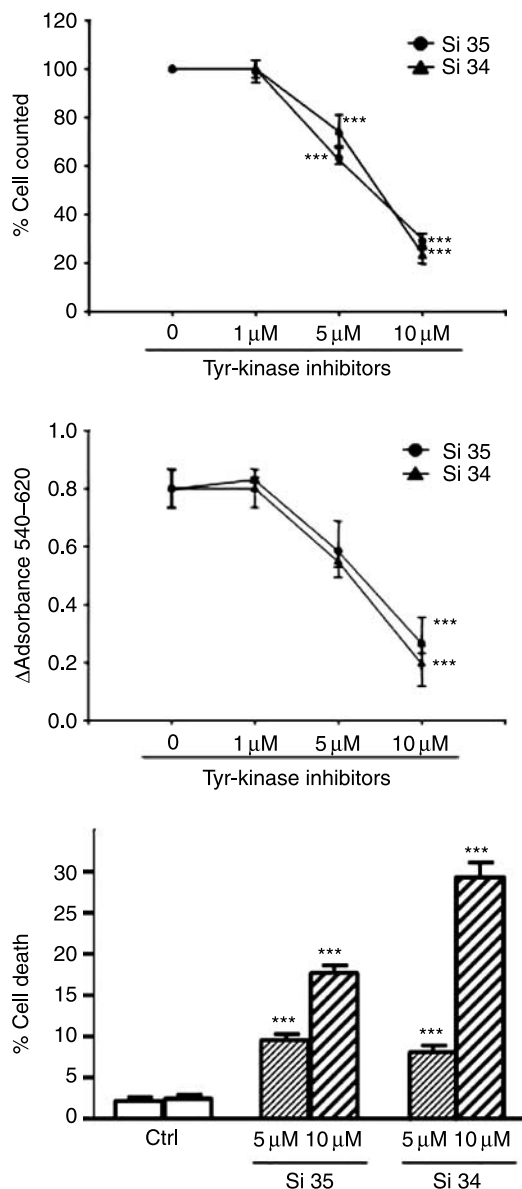


Figure 2 Effects of Si 34 and Si 35 on ARO cells. Upper panel: growth inhibition of ARO cells treated for 72 h with varying concentrations of Si 34 and Si 35. Data represent means \pm s.e.m. of five experiments. Results are expressed as percentage changes with respect to control cell counts (cells treated with corresponding amount of DMSO, see Methods). Middle panel: formazan production (MTT) was measured after 72 h incubation with different concentrations of the compounds. Data represent means \pm s.e.m. of three experiments. As control, cells treated with corresponding amount of DMSO were used (see Methods). Lower panel: mortality rates in ARO cells treated for 72 h with different concentrations of Si 34 and Si 35. Values represent means \pm s.e.m. of at least six different experiments, expressed as percentage of death cells over total counted. As control, cells treated with corresponding amount of DMSO were used (see Methods). * $P < 0.05$; *** $P < 0.001$.

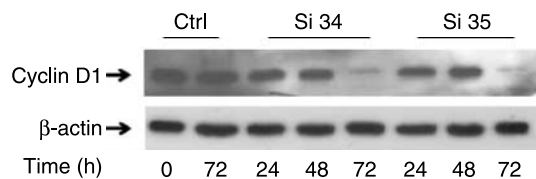


Figure 3 Effects of Si 34 on the Cyclin D1 expression. After 24, 48, and 72 h of treatment with 10 μM Si 34 or Si 35, total proteins were extracted from the cells and immunoblotted using anti-cyclin D1 antibody, as described in Methods. The results are standardized to β-actin protein.

after treatment of ARO cells with 10 μM Si 34 or Si 35 showed a clear reduction of this protein expression, suggesting an arrest in G1–S transition determined by these compounds (Fig. 3).

Effects of Si 34 loaded liposomes on thyroid cancer cells

Next, pegylated unilamellar liposomes were prepared and investigated as potential drug carrier for these pyrazolopyrimidine derivatives in the attempt to improve their biopharmaceutical features, including their water solubility/dispersibility and pharmacokinetic profile and maintaining their capacity to suitably interact with biological substrates. Considering the similar antiproliferative effect of Si 34 and Si 35, only Si 34 loaded unilamellar liposomes were prepared and assayed for the anticancer activity against ARO cells in comparison with the free drug. As shown in Fig. 4, the liposome

formulation significantly improved the antiproliferative activity of Si 34. Namely, a significant inhibition of the cell growth was observed after a shorter exposure time, i.e., 48 h (the free drug exerted its action only after 72 h incubation), with inhibition values of 54% and 13% for 5 and 10 μM respectively. A significant increase of cell mortality was also observed after 48 h incubation with the liposome formulation of Si 34 (17% and 35% at 5 and 10 μM respectively), while after 72 h at 10 μM, almost all the cells were killed (Fig. 4). Thus, an increase of effectiveness was evident with the liposome-encapsulated Si 34 compared with the free drug. The effects of liposomal Si 34 were further investigated in other five thyroid cancer cell lines.

As shown in Fig. 5, almost all the cell lines responded to the drug, with the strongest increase of mortality detected in the ARO cells.

Effects of Si 34 loaded liposomes on apoptosis

We next attempted to determine whether the cytotoxic effects of these compounds involved the induction of apoptosis. Immunoblot analysis of PARP cleavage (Fig. 6a) and fluorescent microscopy analysis of cell nuclei (data not shown) revealed low or no signs of apoptosis after exposure to 10 μM Si 34 or 5 μM liposomal Si 34 (concentrations that reduced cell growth and increased cell mortality). In contrast, the more sensitive FACS analysis using annexin V labeling of the cells revealed the presence of signs of

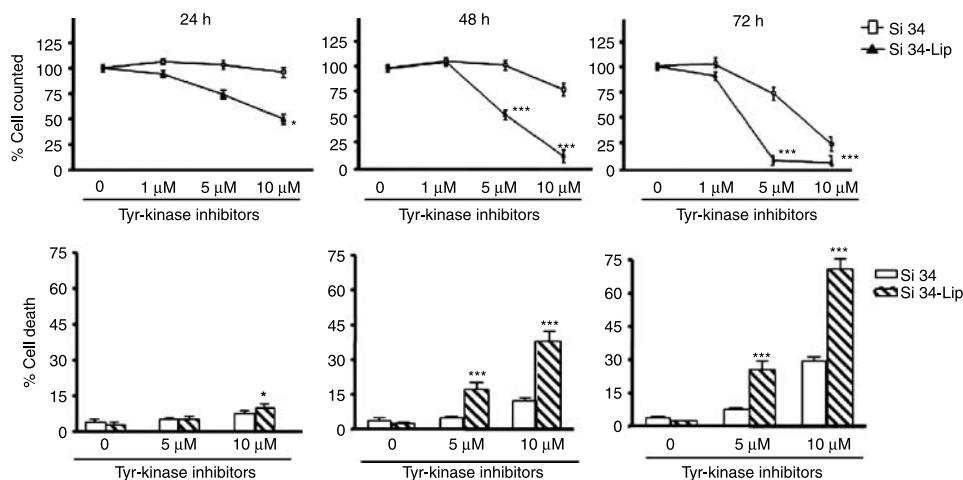


Figure 4 Effects of Si 34 encapsulated in unilamellar liposomes in ARO cells. Upper panel: growth inhibition of ARO cells treated for 24, 48, or 72 h with varying concentrations of Si 34 free (Si 34) or entrapped in liposomes (Si 34-Lip). Data represent means ± s.e.m. of three experiments. Results are expressed as percentage changes with respect to control cell counts (cells treated with corresponding amount of DMSO for free drugs, culture medium for liposome-entrapped preparations, see Methods). Lower panel: mortality rates in ARO cells treated for 24, 48, or 72 h with different concentrations of Si 34 free or entrapped in liposomes. Values represent the mean ± s.e.m. of at least three different experiments, expressed as percentage of death cells over total counted. As control, cells treated with corresponding amount of DMSO for free drugs, culture medium for liposome-entrapped preparations were used (see Methods). **P* < 0.05; ****P* < 0.001 (Si 34-Lip versus Si 34).

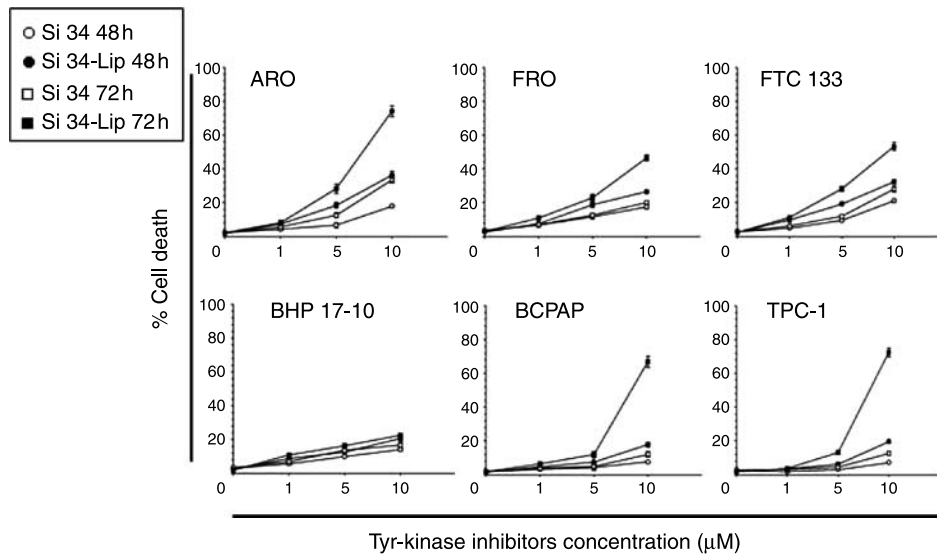


Figure 5 Effects of liposomal Si 34 in six thyroid cancer cell lines. Mortality rates in thyroid cancer cells treated for 48 or 72 h with different concentrations of Si 34 free or entrapped in liposomes (Si 34-Lip). Values represent the mean \pm s.e.m. of at least three different experiments, expressed as percentage of death cells over total counted. As control, cells treated with corresponding amount of DMSO for free drugs, culture medium for liposome-entrapped preparations were used (see Methods). In controls, mortality rate was always under 5% of total.

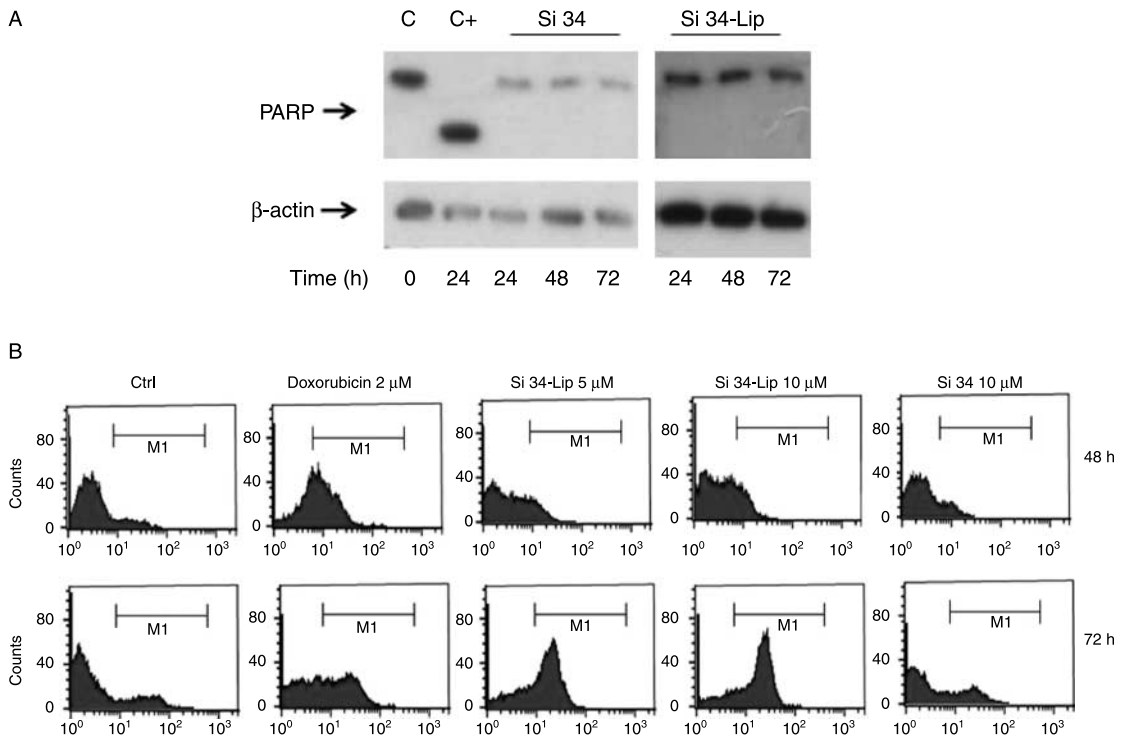


Figure 6 Effects of Si 34 on apoptosis. (A) No evidence of PARP cleavage after treatment of ARO cells with Si 34 or liposomal-entrapped Si 34 (Si 34-Lip). Cells were incubated for 24, 48, and 72 h with Si 34 or Si 34-Lip 5 μ M/l. As control, cells treated with corresponding amount of DMSO were used (C). Total proteins were extracted and analyzed by means of immunoblotting with PARP antibody, as described in Methods. The cleaved 86 kDa fragment of PARP could not be detected. ARO cells treated for 24 h with 10 nM paclitaxel/doxorubicin were used as positive controls (C+). Results were standardized to β -actin protein. (B) Annexin V (FITC) staining of ARO cells after treatment with Si 34 free or entrapped in liposomes (Si 34-Lip). After 48 h of treatment with the drugs or a solution of 2 μ M/l doxorubicin as positive control, analysis was performed using flow cytometry, as described in Methods.

apoptosis after Si 34 treatment and more evident presence of apoptosis with the liposomal preparation (Fig. 6b). Therefore, ARO cells undergo apoptosis after treatment with these tyrosine kinase inhibitors, but apoptosis cannot fully explain the toxic effects of the compounds.

Effects of Si 34 loaded liposomes on invasive-ness and signal transduction pathways

We next investigated the effects of these compounds on EGF-induced migration and the activation of c-src and ERK phosphorylation. Using a Matrigel invasiveness assay in ARO cells treated with 50 ng/ml EGF, we found a significant reduction of percent invasion after 48 h incubation with 5 μ M of the liposomal Si 34 (Fig. 7). Time-course experiments revealed an early inhibition of both c-src and ERK phosphorylation using the liposomal Si 34 in ARO cells incubated with EGF. As shown in Fig. 8, the drug treatment decreased, after 1 h incubation, the levels of the phosphorylated kinases without affecting the content of non-phosphorylated proteins.

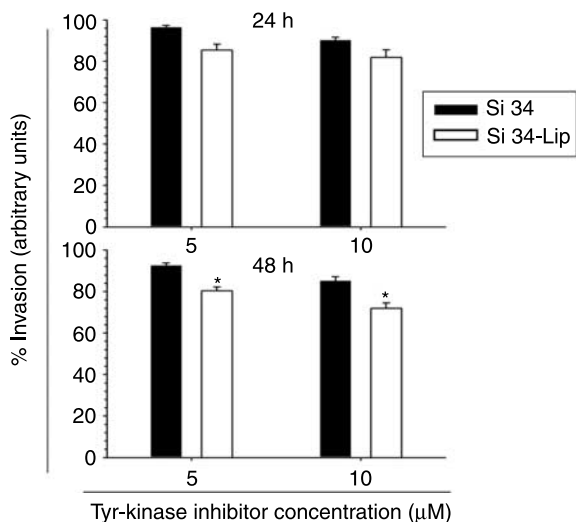


Figure 7 Effects of Si 34 loaded liposomes on cell invasiveness. ARO cells were allowed to cross a matrigel-coated 8 μ m pore size polycarbonate filters in response to EGF (50 ng/ml), used as the chemoattractive molecule in the lower compartment of the chamber in the presence or not of free or liposomal-entrapped Si 34. After the incubation periods, the upper surface of the filter was wiped off, and the remaining cells that traversed the Matrigel and spread on the lower surface of the filter were collected separately and an MTT assay was then performed. The percentage of cell viability of each chamber was calculated with respect to the control (untreated cells). A representative of three experiments performed in triplicate is shown. * $P < 0.05$.

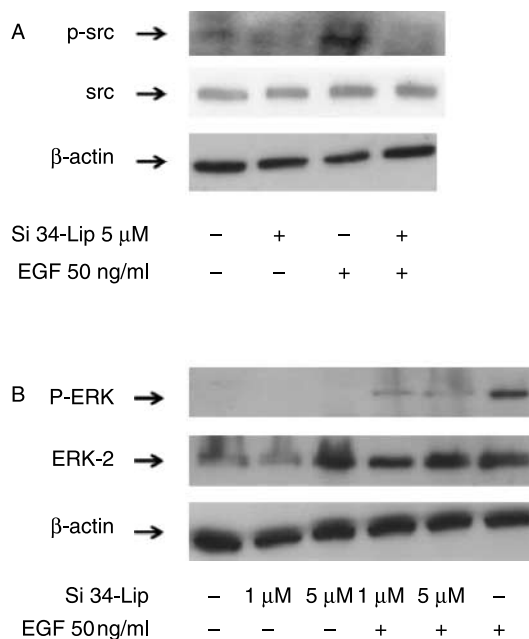


Figure 8 Effects of Si 34 loaded liposomes on src and ERK phosphorylation. (A) After 60 min of treatment with 5 μ M/l of the drug, in the presence or not of 50 ng/ml EGF, total proteins were extracted from the cells and immunoblotted using anti-src and anti-p-src antibody, as described in Methods. The results are standardized to β -actin protein. (B) Two concentration of liposomal Si 34 (1 and 5 μ M/l) were incubated for 60 min with ARO cells in the presence or not of 50 ng/ml EGF. After total protein extraction, immunoblot analysis was performed using an anti-ERK and anti-p-ERK antibody, as described in Methods. The results are standardized to β -actin protein. A representative of three experiments is shown.

Effects of Si 34 loaded liposomes on the growth of xenograft tumors

We tested the ability of the liposomal Si 34 to kill ARO cells growing *in vivo*. For this purpose, immunodeficient mice were injected with 10^6 cancer cells in one flank and treated with 25 or 50 mg/kg (five mice for group) of the drug i.v. after the tumor reached approximately a diameter of 10 mm. The control group (five mice) was injected only with empty liposomes. The growth of tumors was measured at regular intervals, as described in Methods. Figure 9a shows the mean increase in tumor size for each group. As shown in Fig. 9b, after 3 weeks of treatment, the control mice had obvious large tumors, whereas the Si 34 treated animals, especially those which received 50 mg/kg per die of the drug, possessed much smaller tumors. No significant effect on body weight, feeding behavior and motor activity was observed in the treated mice. A lower inhibitory effect on the tumor growth was observed also in a group of mice treated with the free Si 34 drug dissolved in DMSO (data not shown).

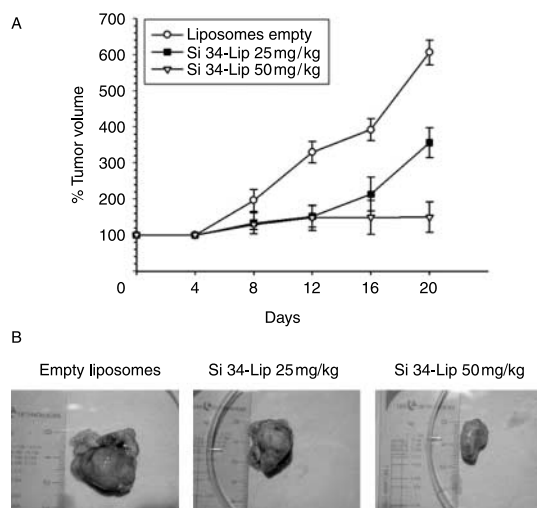


Figure 9 Effects of Si 34 on xenograft tumors. Immuno-deficient mice with ARO cancer cells were treated for 3 weeks with 25 or 50 mg/kg per day liposomal-Si 34 by i.v. injections. (A) Growth of tumors during course of experiments as measured by increase in calculated volume size of individual tumors. (B) Representative tumor from control and Si 34 treated cohorts.

Discussion

Treatment of anaplastic thyroid carcinomas is still a major clinical challenge. These tumors, which are unable to concentrate radioiodine, are generally managed with a combination of aggressive surgery, external beam radiation, and chemotherapy. Unfortunately, this approach is rarely effective, particularly when metastases have developed (Ain 1998, Pasieka 2003). The search continues for new and more effective strategies for treating these tumors, including gene therapy-based approaches and novel drugs with improved selectivity, bioavailability, and/or pharmacokinetic properties. There are a number of well-established human thyroid cancer cell lines that can be used for preclinical *in vitro* testing of candidate molecules with potential antitumoral activity against these tumors. The ARO cell line (Fagin *et al.* 1993) is the most widely used model for this purpose, and the growth of these cells is reportedly inhibited by several putative anticancer drugs (Braga-Basaria *et al.* 2004, Nobuhara *et al.* 2005, Copland *et al.* 2006, Luong *et al.* 2006, Ouyang *et al.* 2006).

In recent years, increasing attention has been focused on tyrosine kinase (TK) inhibitors as potential anticancer drugs. Excellent results have been obtained with Imatinib (Gleevec) in chronic myeloid leukemia either *in vitro* or, more importantly, *in vivo* in studies conducted in humans (May 2003). This experience has led to the development of other molecules of this type,

whose potential antitumoral effects are currently being evaluated in preclinical and clinical studies. The cytoplasmic protein c-src is a ubiquitous non-receptor TK that is overexpressed or abnormally activated in several types of tumors (Bjorge *et al.* 2000). It has been identified as a potential target of selective TK inhibitors. Two such agents, the pyrazolopyrimidine derivatives PP1 and PP2, have been shown to inhibit the kinase activity of the RET oncogene and exert negative effects on the growth of medullary thyroid tumor cells *in vitro* and *in vivo* (Carlomagno *et al.* 2002, 2003). These data led to the synthesis of a series of compounds with the same structural backbone as that of PP1 and PP2. When tested in epithelial tumor cell lines, these compounds appeared to be more potent than PP2 in inhibiting src phosphorylation and cell proliferation (Carraro *et al.* 2004, Schenone *et al.* 2004b, Angelucci *et al.* 2006, Morisi *et al.* 2007).

In the present study, the two most effective compounds of this series were investigated to determine whether they exert antiproliferative effects in anaplastic human thyroid cancer cells. Our data demonstrate that the pyrazolopyrimidine derivatives Si 34 and Si 35 significantly inhibited growth and increased death rates in ARO cells. Similar effects have been observed in other epithelial tumor cell lines; in those cases, the cell death induced by Si 34 and Si 35 was clearly apoptotic (Carraro *et al.* 2004, Schenone *et al.* 2004b), whereas in the present study there was no evidence of apoptosis according to experiments of PARP cleavage and staining of cell nuclei, whereas apoptotic cells were barely detectable using the more sensitive FACS analysis. It is likely that Si 34 and Si 35 provoke both necrotic and apoptotic death in this thyroid cancer cell line and that a partial resistance to apoptosis could be related to the presence of p53 mutations (Fagin *et al.* 1993). Our results also demonstrate that Si 34 and Si 35 downregulated cyclin D1 protein in ARO cells. Cyclin D1 is a rate-limiting factor in cell proliferation induced by growth factors in various models and is often overexpressed in cancer cells, including anaplastic thyroid tumors (Pestell *et al.* 1999, Wiseman *et al.* 2007). A decrease of cyclin D1 levels has also been described in association with growth arrest of ARO cells determined by antiproliferative drugs such as PPAR γ agonists or suberoylanilide hydroxamic acid (Aiello *et al.* 2006, Luong *et al.* 2006).

An important issue to be fulfilled for a potential *in vivo* application in cancer chemotherapy of a novel agent effective *in vitro* at relatively high dosage is represented by the design of a suitable drug dosage form that is able to allow a certain exposition time of the cancer tissue, by

ensuring a prolonged blood half-life, and avoid toxic effects due to its biocompatibility. In the case of hydrophobic drugs with a low water solubility, i.e., Si 34 and Si 35, the use of DMSO (widely used in cell-culture studies) as solubilizing agent would be not suitable for clinical settings. For this reason, a liposome formulation of Si 34 was prepared and proposed as a possible drug carrier for both *in vitro* and *in vivo* applications. Our previous results (Celano et al. 2004, Calvagno et al. 2007) showed that the liposomal carrier may provide even *in vitro* effective advantages over free drugs in terms of both dose-dependent and exposition time-dependent anticancer effect. In the perspective of a possible *in vivo* application, pegylated liposomes were prepared and, hence, the presence of polyethylene glycol (PEG) moieties on the surface of liposomes may promote the carrier bioadhesion at the level of cellular surface, thus stimulating a liposome–cell interaction (Sadzuka & Hirota 1998, Sadzuka et al. 2003), i.e., liposome uptake into tumor cells, inter-membrane drug passage, and/or microenvironments with high local drug concentrations that favor intracellular drug entrance (Calvagno et al. 2007).

Our data demonstrate that in the case of pyrazolopyrimidine derivative Si 34, liposomes improved the *in vitro* activity of the drug most probably by enhancing the drug delivery at the level of tumor cells. Moreover, the liposomal preparation of Si 34 was effective also in arresting the growth of xenograft tumors *in vivo*. As observed in other epithelial tumor cell lines for this and other pyrazolopyrimidine derivatives (Carraro et al. 2004, Schenone et al. 2004b, Angelucci et al. 2006, Donnini et al. 2006), the effect of liposomal Si 34 was associated with inhibition of src and ERK phosphorylation and a partial reduction on the migration ability of the tumor cells. These effects are obtained using a concentration of the liposomal drug in a similar range of that described for other agents tested in the same or similar *in vitro* and *in vivo* models (Carlomagno et al. 2003, Nobuhara et al. 2005, Luong et al. 2006, Ball et al. 2007), targeting the same (ERK-mediated) growth-stimulatory pathway, demonstrated to be activated in poorly differentiated and anaplastic thyroid cancer. For some of these novel drugs, clinical trials are currently in progress, alone, or in combination with conventional anticancer drugs (Kundra & Burman 2007).

The promising results of the present study confirm the validity of approaches that target src-related tyrosine kinase activity for arresting the growth of anaplastic thyroid tumors and indicate that Si 34 and Si 35 are potentially effective drugs for this purpose. A unilamellar-pegylated liposome formulation, which represent a non-toxic alternative to DMSO or any other organic solvent and/or surfactant, has also been set up as a

potential carrier in view of an *in vivo* use of the compounds. The possibility, already explored in other models (Tardi et al. 2007), to use the liposome as a multidrug carrier, may provide further advantages in view of clinical trials using combined therapies for the treatment of anaplastic thyroid tumors.

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