Plasmin-Binding Tripeptide-Decorated Liposomes Loading Pyrazolo[3,4-*d*]pyrimidines for Targeting Hepatocellular CarcinomaPierpaolo Calandro,^{†,‡,○} Giulia Iovenitti,^{†,○} Claudio Zamperini,^{†,§} Francesca Candita,[†] Elena Dreassi,^{†,Ⓛ} Mario Chiariello,^{‡,Ⓛ} Adriano Angelucci,^{||} Silvia Schenone,[⊥] Maurizio Botta,^{*,†,§,Ⓛ,Ⓢ} and Arianna Mancini^{†,▽}[†]Dipartimento Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via A. Moro 2, I-53100 Siena, Italy[‡]Consiglio Nazionale delle Ricerche, Istituto di Fisiologia Clinica and Istituto per lo Studio, la Prevenzione e la Rete Oncologica (ISPRO), Core Research Laboratory, Via Fiorentina 1, 53100 Siena, Italy[§]Lead Discovery Siena S.r.l., Via Vittorio Alfieri 31, 53019 Castelnuovo Berardenga, Siena, Italy^{||}Dipartimento di Scienze Cliniche Applicate e Biotecnologiche, Università degli Studi dell'Aquila, Via Vetoio, 67100, Coppito, L'Aquila, Italy[⊥]Dipartimento di Farmacia, Università degli Studi di Genova, Viale Benedetto XV, 3, 16132 Genova, Italy[Ⓢ]Biotechnology College of Science and Technology, Temple University, Biolife Science Building, Suite 333, 1900 N 12th Street, Philadelphia, Pennsylvania 19122, United States[▽]Dipartimento di Farmacia, Università di Pisa, Via Bonanno Pisano 6, 56126 Pisa, Italy

S Supporting Information

ABSTRACT: Hepatocellular carcinoma (HCC) is one of the most fatal cancer types worldwide. HCC cells were proved to overexpress c-Src and Sgk1, a tyrosine and a serine-threonine kinase, respectively, whose role is crucial for the development and progression of the tumor. Pyrazolo[3,4-*d*]pyrimidine derivatives are a class of tyrosine kinase inhibitors that have shown good activity against HepG2. HCC cells were also proved to overexpress plasmin, which is localized on the cell surface bound to its receptors. In this study, a tripeptide with sequence D-Ala-Phe-Lys, which binds a specific reactive site of plasmin, was synthesized and characterized. This tripeptide was used to decorate liposomes encapsulating three selected pyrazolo[3,4-*d*]pyrimidines. Liposomes bearing tripeptide have been characterized, not showing remarkable differences with respect to the corresponding tripeptide-free liposomes. *In vitro* HepG2 cell uptake profiles and cytotoxicities showed that the presence of the tripeptide on the liposomal membrane surface improves the cell-penetrating ability of liposomes and increases the activity of two of the three tested compounds.

KEYWORDS: Hepatocellular carcinoma, active-targeted liposomes, pyrazolo[3,4-*d*]pyrimidines, plasmin

Hepatocellular carcinoma (HCC) is the most common histological subtype among primary liver malignancies and one of the most fatal cancer types in the world.¹ For intermediate and advanced stage (70% of HCC patients), there are palliative treatments including transarterial chemoembolization (TACE). Sorafenib, an oral multitargeted kinase inhibitor, has been approved for the treatment of HCC.² Despite the available treatments, the mean survival rate of patients with advanced stage HCC is less than 1 year.³ As in many other cancer types,⁴ both moderately and well differentiated HCC cells were proved to overexpress c-Src family proteins,^{5,6} a class of nonreceptor tyrosine kinases involved in cascades of signaling pathways essential for cell proliferation, motility, and survival. c-Src hyperactivation plays a central role in HCC progression and may be partly responsible for the malignant transformation of hepatocytes.⁶ Accordingly, c-Src inhibitors have the potential to

be used as antineoplastic agents for HCC.⁷ Pyrazolo[3,4-*d*]pyrimidine derivatives are a class of small molecules ATP-competitive c-Src inhibitors, which have been developed, synthesized, and extensively studied in our laboratories. They were found to induce apoptosis and proliferation impairment in several solid cancer cell lines.^{8–11} Furthermore, one of these molecules, named SI113, was identified as a potent inhibitor of both c-Src and Sgk1, a Ser/Thr kinase that has recently gained attention as molecular target in oncology for its regulating role in cell survival, proliferation, and differentiation.^{12,13} In HCC, Sgk1 expression is particularly relevant, and its inhibition by SI113 in HepG2 and HUH-7 cell lines resulted in a dramatic increase in

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Figure 1. Molecular structures of compounds SI113, S29, and SI163, encapsulated into liposomes and TRP (plasmin-binding tripeptide-linker), used to decorate liposomal surface.

Table 1. Compounds S29, SI113, SI163: activity against Src and Sgk1, ADME Properties, and IC₅₀ against HepG2 Cells

compd	Src (K_i , μM) ^a	Sgk1 (residual activity %) ^b	aq solub ($\mu\text{g mL}^{-1}$) ^c	Papp ^d (10^{-6} cm/sec 50% DMSO)	met stab % ^e	IC ₅₀ (μM)
S29	0.8		<0.04	16.6	94.0	1.6 $\pm$ 1.1
SI113	2.4	0	0.4	2.6	$\geq$ 95.0	12.5 $\pm$ 3.4
SI163	0.0093		0.098	10.0	93.6	3.2 $\pm$ 2.3

^aAll data were previously reported.^{11,36} ^bResidual activity of Src kinase after exposure to 12.5 μM SI113 for 30 min.¹³ ^cAqueous solubility was determined by means of the LC-UV method. ^dApparent permeability was determined with PAMPA assay. ^eThe human liver microsomal stability is expressed as percentage of unmodified parent drug.

apoptosis and altered cell cycle. In human hepatocarcinoma cells xenografted immunodeficient mice, SI113 arrested cancer growth, inducing high levels of necrosis in tumor tissues.¹⁴

However, the main weakness of these molecules is their low aqueous solubility, which could give rise to low bioavailability and low target specificity, affecting their pharmacologic activity. To overcome this issue, liposomal technology has recently been explored: four pyrazolo[3,4-*d*]pyrimidine derivatives with antineuroblastoma activity have been encapsulated in stealth liposomes and tested *in vivo* with an improvement in pharmacokinetic properties and *in vitro* with retention of cytotoxic efficacy with respect to free compounds in human neuroblastoma cell line.¹⁵ Liposomes have been extensively investigated and used as pharmaceutical carriers for antineoplastic drugs for their many favorable physicochemical and pharmacological features and their versatility.¹⁶ Ligand-conjugated liposomes for active targeting offer the prospect of improving the effectiveness and minimizing the off-target effects of anticancer therapy.¹⁷ Active delivery of liposomes may provide increased accumulation at the target tissue, enhanced cellular internalization, and triggered release of therapeutic payloads, exploiting pathological peculiarities in the tumor's microenvironment.¹⁸

It is widely acknowledged that the broad-spectrum serine protease plasmin (PM) and its activation system have a primary role in cancer metastasis.¹⁹ PM is partly responsible, directly or by activating several pro-metalloproteases (MMPs), for the degradation of extra-cellular matrix (ECM), disaggregation of basement lamina, and catabolism of adhesion molecules, promoting cancer cells invasion. PM is mostly found in its cell surface-associated form, bound to its receptors (plasminogen receptors, PG-R),^{20,21} which are expressed by a wide variety of cells with high density ($>10^7$ binding sites for plasminogen on some cells),²² especially carcinoma cells.²³ This allows for localized and much greater proteolytic activity and protection from inactivation (active PM occurs in the blood circulation in very low concentrations because it is rapidly inhibited by α -antiplasmin). Some of PG-R are overexpressed on carcinoma

cells, among which are α -enolase (ENO-1),^{24,25} cytotkeleton 8,²⁶ annexin A2,²⁷ amphotericin.²⁸ ENO-1, in particular, was found to be overexpressed in more than 20 types of human cancer.²⁹ The imbalance between plasminogen activators (PAs) and PM in tumor microenvironment is responsible for the excessive production of PM. Increased expression level of plasminogen activators has been recognized in many types of malignant tumors,^{30–32} including HCC.³³

The first part of this work describes the synthesis and the characterization of a specific tripeptide with sequence D-Ala-Phe-Lys, which binds a specific reactive site of plasmin.³⁴ The second part focuses on the functionalization of the outer membrane of pyrazolo[3,4-*d*]pyrimidine-loaded liposomes with the plasmin-binding tripeptide. The physicochemical properties (size, structure, surface) of pyrazolo[3,4-*d*]pyrimidine-loaded bare liposomes (LP) and pyrazolo[3,4-*d*]pyrimidine-loaded tripeptide-conjugated liposomes (TLP) will be examined and compared. Finally, the *in vitro* uptake profiles and cytotoxicity of free drugs and the drugs entrapped in liposomes with and without plasmin-binding tripeptide were examined on the HepG2 HCC cell line.

Synthesis and characterization of pyrazolo[3,4-*d*]pyrimidine compounds (S29, SI113, SI163) were previously reported by us.^{10,35,36} Structures of compounds have been reported in Figure 1, as well as the tripeptide-linker (TRP) structure. ADME properties, activity against Src and Sgk1, and IC₅₀ against HepG2 cells of S29, SI113, and SI163 have been reported in Table 1.

The tripeptide-linker (TRP) was designed and synthesized in good yields, developing a simple liquid phase method-based procedure. In order to synthesize the tripeptidyl portion, it was pivotal to set up an orthogonal protection strategy. The synthetic pathway is summarized in Scheme 1. H-Lys(Z)-OH methylester hydrochloride, obtained by treatment of H-Lys(Z)-OH (1) with SOCl₂ in MeOH, was coupled with Boc-Phe-OH using EDC·HCl/DMAP as an activating system. Dipeptide 3 was then N-deprotected with 10% TFA affording 4, which was coupled with Boc-D-Ala-OH in the presence of EDC·HCl/DMAP. The carbobenzyloxyl moiety was then cleaved by hydrogenation. In

Scheme 1. Preparation of Tripeptide Portion D-Ala-Phe-Lys^a

^aReagents and conditions: (a) SOCl₂, MeOH, r.t., 16 h; (b) Boc-Phe-OH, DMAP, EDC·HCl, CH₂Cl₂ dry, r.t., 16 h; (c) 10% TFA, CH₂Cl₂ dry, r.t., 1 h; (d) Boc-D-Ala-OH, DMAP, EDC·HCl, CH₂Cl₂ dry, r.t., 16 h; (e) H₂, Pd/C 10%, MeOH, HCl conc., r.t., 12 h; (f) Boc₂O, THF, 5% NaOH, r.t., 16 h.

conclusion, after pH adjustment to 5, the reaction of the tripeptidyl intermediate 6 with Boc₂O and 5% NaOH led to the formation of the desired tripeptide D-Ala-Phe-Lys (7).

Therefore, a linker H₂N-CH₂-CH₂-SH was introduced at the C-terminal tripeptide sequence by amide bond formation in order to activate the peptide toward maleimide liposomes (Scheme 2). The 2-aminoethanethiol (8) hydrochloride

Scheme 2. Preparation of TRP^a

^aReagents and conditions: (g) 3,4-dihydro-2H-pyran, *p*-TsOH cat, CH₂Cl₂ dry r.t., 5 h; (h) D-Ala-Phe-Lys (7), DMAP, EDC·HCl, CH₂Cl₂ dry, r.t., 16 h; (i) 10% TFA, CH₂Cl₂ dry, r.t., 16 h.

commercially available was SH-protected with 3,4-dihydro-2H-pyran (9). After, linker 9 was coupled with tripeptide 7 using the same condensing agents reported above. Finally, all protecting groups were removed by a single step with 10% TFA providing the final product TRP (11). This approach allowed preparing tripeptide-conjugated liposomes by coupling the free thiol group of TRP and the maleimide terminus of DSPE-PEG2000-maleimide.

Further details regarding materials, synthesis, and NMR characterization of TRP are reported in the Supporting Information.

TRP was ionized and fragmented by LC-MS/MS with progressively higher collision energy to produce spectra that were used for its identification and characterization. In Figure 2, a schematic representation of the TRP sites that undergo MS/MS fragmentation is illustrated. Being the weakest, peptide bonds CO-NH require low collision energy (−20, −15, and −10 V) to

Figure 2. Schematic representation of TRP ($R_1 = \text{CH}_3$; $R_2 = \text{CH}_3\text{-Bn}$; $R_3 = \text{CH}_3\text{-(CH}_2\text{)}_4\text{-NH}_2$) and the sites that undergo MS/MS fragmentation, their m/z (black), and the corresponding collision energy in volts (red).

be fragmented and produced ions with m/z 276.17, 353.20, 219.11, 206.13, and 129.10. The 191.12 and 100.07 m/z fragments derived from the rupture of the same bond, as well as 219.11 and 206.13. Details about the structures of the fragments are reported in the Supporting Information.

S29, SI113, and SI163 were chosen from the wide pyrazolo-[3,4-*d*]pyrimidine derivatives library to be formulated as targeting liposomes against HCC. K_i values for Src in the submicromolar range and ADME parameters have been the main factors determining the selection. Particular focus has been paid to SI113 because of its inhibitory activity against both c-Src and Sgk1, its effectiveness in inducing apoptosis in HepG2 cell line and in reducing tumor growth in HepG2 xenografted mice.¹⁴

With the aim of improving their ADME profile and enhancing their therapeutic effectiveness, a stealth liposomal formulation has been developed. S29-LP, SI113-LP, and SI163-LP have been synthesized by thin layer evaporation method from DPPC/CHOL/DPPE-PEG2000, 2:1:0.1 molar ratio. Each compound (S29, SI113, SI163) was added to the lipid mixture to obtain a final concentration of 0.8 mM in the final volume. The dry lipid film was hydrated in 25 mM HEPES and 140 mM NaCl buffer, pH 7.4, at 60 °C. The downsizing of large multilamellar vesicles (MLV) was achieved using sonic energy, reducing dimension and lamellarity to produce small unilamellar vesicles (SUV). The liposomal suspensions were filtered through 0.2 μm filters to exclude oversized liposomes that may undergo more rapid and extensive opsonization after intravenously injection, inducing their elimination from the blood circulation by the mononuclear phagocyte system (MPS).³⁷ Mean diameters, ζ -potentials, and PDI were measured by dynamic light scattering (DLS), and results are shown in Table 2. Size and ζ -potential values for S29-LP, SI113-LP, and SI163-LP were 153.7, 111.6, 119.3 nm and −29.7, −47.7, −38.7 mV, respectively. These results suggest that the obtained liposomes were stable and did not have the tendency to form aggregates, increasing in dimensions, thus being optimal for *in vivo* administration. Indeed, ζ -potential values lower than −25 mV or greater than +25 mV indicate an electrostatic repulsion sufficient to maintain separation between particles.³⁸ Drug encapsulation efficacy % was determined by LC-UV-MS (for methods, see Supporting Information) after the lysis of liposomes by adding ethanol to the suspensions, and results have been reported in Table 2. EE% ranged from 44% (S29) to 83% (SI163) and 95% (SI113). S29 showed a lower EE % probably as a consequence of its high hydrophobicity and of the competition with cholesterol for the hydrophobic space in the lipid bilayer.³⁹

Table 2. Properties of Bare Liposomes and TRP-Conjugated Liposomes Encapsulating Pyrazolo[3,4-*d*]pyrimidines

formulation	S29-LP	S29-TLP	SI113-LP	SI113-TLP	SI163-LP	SI163-TLP
EE % ^a	44 ± 7.5	31 ± 8.3	95 ± 1.5	94 ± 3.2	83 ± 6.5	98 ± 1.2
size (nm) ^b	153.7 ± 5.4	103.4 ± 6.67	111.6 ± 12.6	118.3 ± 6.7	119.3 ± 6.5	121.9 ± 11.76
PDI ^b	0.679 ± 0.034	0.183 ± 0.012	0.539 ± 0.058	0.228 ± 0.043	0.813 ± 0.114	0.317 ± 0.084
ζ potential (mV) ^b	−29.7 ± 3.48	−20.1 ± 4.36	−47.7 ± 4.79	−36.3 ± 5.25	−38.7 ± 4.28	−15.4 ± 6.12
IC ₅₀ (μM)	1.6 ± 1.2	1.8 ± 1.1	8.0 ± 3.5	2.5 ± 1.9	1.8 ± 1.4	1.0 ± 1.1

^aFor experimental details see [Supporting Information](#). ^bDetermined by DLS.

Figure 3. Left: Fluorescence microscopy images of HepG2 cells following LPs and TLPs administration: cells after 5 min of incubation with LPs (A) and TLPs (B) and cells after 1 h of incubation with LPs (C) and TLPs (D). Green, liposomes; red, cell marker; blue, nuclei. Right: Relative fluorescence intensity per cell area histograms for each formulation.

S29-TLP, SI113-TLP, and SI163-TLP dried lipid films have been prepared by the method previously described for LPs, except for the addition of DSPE-PEG2000-maleimide to the lipid mixture (DPPC/CHOL/DPPE-PEG2000/DSPE-PEG2000-Mal, 2:1:0.08:0.02 molar ratio). After the preparation of the lipid film and its hydration, the sonication and the filtration of the liposomal suspensions, surface decoration of liposomes was achieved by coupling the linking portion of the tripeptide to the maleimide terminus of PEG chains laying on the outer liposomal membrane. The coupling reaction between liposomes and TRP was run at a molar ratio of TRP to DSPE-PEG2000-Mal of 1:4 overnight at 4 °C. The amount of TRP conjugated to the liposomal membrane was determined after the lysis of liposomes by subtracting free TRP from the initial TRP concentration. The 33% of the total amount of TRP effectively bound the liposomal surface, and about 300 tripeptide molecules per liposomes were estimated. Uncoupled TRP was separated from the liposomal suspension by dialysis. TRP-liposomes have been characterized by DLS and HPLC-MS without remarkable differences with respect to bare liposomes (Table 2). Membrane ζ-potential values were measured to be −20.1 mV for S29-TLP, −36.3 mV for SI113-TLP, and −15.4 mV for SI163-TLP. Membrane ζ-potentials for each TLP increased with respect to the corresponding LP. This enhancement is due to the positive net charge possessed by the tripeptide that induces a lower electrostatic repulsion between particles. Nevertheless, particle sizes were measured to be 103.4 nm for S29-TLP, 118.3 nm for SI113-TLP, and 121.9 nm for SI163-TLP, which are excellent dimensional parameters for a future intravenous administration. Moreover, PDI values demonstrated an improvement in homogeneity and uniformity of particles size distribution of TRP-liposomes with respect to TRP free-liposomes. Indeed, TRP-liposomes showed a PDI around 0.2–0.3, indicating a relatively monodispersed particle population, whereas TRP free-liposomes showed PDI values >0.5, suggesting the presence of a broad particle size distribution. Encapsulation efficacy % was greater than 90% for SI113 and SI163 TRP-liposomal formulations (94% and 98%, respectively) and 31% for S29-TLP.

The *in vitro* antiproliferative effects of anticancer pyrazolo[3,4-*d*]pyrimidines-loaded TRP-conjugated liposomes, the respective bare liposomes, and free drugs dissolved in DMSO were evaluated by cytotoxicity assay and determination of cell survival rates by using HepG2 HCC cell line (for materials and methods, see [Supporting Information](#)). Results, shown in Tables 1 and 2, indicate that S29 activity is not significantly altered when the compound is delivered by liposomes, both TRP-conjugated and TRP-free, while SI113 and SI163 displayed a higher cytotoxic activity when delivered by liposomes compared to free compounds. Moreover, for these two molecules, the presence of the tripeptide on the liposomal membrane surface increased the activity of the liposomes-delivered compounds: SI113-TLP resulted 3 times more active than SI113-LP and 5 times more than free compounds, and the cytotoxicity of SI163-TLP was almost 2 times greater than SI163-LP and 3 times greater than free SI163. All the free compounds and the respective liposomal formulation did not show cytotoxicity in normal cells (RWPE-1 cell line) in the concentration range of their IC₅₀ values for HepG2. The only formulation that provoked a cellular mortality greater than 20% was TRP-liposomal S29. Cytotoxicity of empty TRP-liposomes and bare liposomes was evaluated on HepG2, and both were proved to be safe. These outcomes proved that the intracellular delivery of the anticancer pyrazolo[3,4-*d*]pyrimidines was enhanced by plasmin-binding tripeptide-conjugated liposomes with an increased amount of drug available to act against cancer cells. The fact that S29 activity was not enhanced by the inclusion of the molecule in the liposomal structure is probably due to the poor encapsulation efficacy % that results in a lower concentration of compound per single carrier.⁴⁰ Both for the low EE% and the cytotoxicity tests results, S29 has been considered unpromising, and it will not be selected for future studies.

In vitro cellular uptake assay was performed. Fluorescent TRP-liposomes and bare liposomes were prepared, incorporating a fluorescent phospholipid (PE-CF) in the lipids mixture and subsequently administered to HepG2 cells (see [Supporting Information](#)).

After 5 min and 1 h from the administration, cells treated by both the liposomal formulations were observed by confocal fluorescence imaging. Z-stack images and histograms indicating relative fluorescence intensity per cell area for each formulation are shown in Figure 3 (fluorescence values have been extrapolated from the images by the software ImageJ): HepG2 cells treated with TRP-liposomes displayed a higher fluorescence (Figure 3B,D) than the cells treated with free TRP liposomes (Figure 3A,C). Images suggest that the presence of the PM-binding tripeptide on the liposomal surface allowed an improved and more rapid interaction with the cellular membrane and subsequent internalization, confirming the evidence that a greater amount of anticancer drug was taken up by the cells, being more effective.

In summary, first we synthesized and characterized a tripeptide with a specific sequence able to bind cell-associated PM that is proven to be overexpressed in HCC cells. After full characterization of TRP via collision-induced dissociation by tandem mass spectroscopy, we developed three liposomal carrier systems that were encapsulated with a pyrazolo[3,4-*d*]pyrimidine compound each (S29, SI113, SI163) and decorated with the PM-binding tripeptide, with a density of ~300 molecules of tripeptide per liposome. Encapsulation efficacy % was greater than 90% for SI113 and SI163 formulations and 31% for S29; particles size was measured to be around 100 nm for all the formulations and membrane ζ -potential values ranged from -36.3 to -15.4 mV. In general, all the tripeptide-liposomal formulations have shown no remarkable differences in characterization profiles with respect to the corresponding bare liposomes (except for an enhancement in ζ -potential values due to the positive charge of TRP and an improvement in PDI values) and present good parameters for future *in vivo* administration. The *in vitro* antiproliferative effects of anticancer pyrazolo[3,4-*d*]pyrimidines-loaded tripeptide-conjugated liposomes were then evaluated on HepG2 cells and both SI113 and SI163 tripeptide-liposomes resulted more active than bare liposomes and free compounds. This outcome suggests an enhancement in cell-penetrating ability provided by the presence of the tripeptide on the liposomal surface and consequently a greater amount of drug delivered into the cancer cells, which suppressed proliferation and survival. This evidence was confirmed by confocal microscopy tests that showed a greater and more rapid cellular internalization, promoted by the interaction between the tripeptide and cell surface-associated PM. All these results taken together represent a very promising starting point for future *in vivo* pharmacokinetic and pharmacodynamic studies.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00062.

Reagents, synthesis, NMR, LC–MS and MS/MS characterization of TRP; materials and experimental details for preparation and characterization of liposomes and for cellular assays, and instruments details (PDF)

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Notes

The authors declare no competing financial interest.

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