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Optimizing tumor targeting of the lipophilic EGFR-binding radiotracer SKI243 using a liposomal nanoparticle delivery system

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Abstract

Positron emission tomography (PET) of epidermal growth factor receptor (EGFR) kinase-specific radiolabeled tracers could provide a means for non-invasively characterizing EGFR expression and signaling activity in patients' tumors before, during, and after therapy with EGFR inhibitors. Towards this goal, our group has developed PET tracers which irreversibly bind to EGFR. However, tumor uptake is relatively low because of both the lipophilicity of such tracers (e.g. the morpholino-[124I]-IPQA [SKI212243]), with octanol-to-water partition coefficients of up to 4, and a short dwell time in the blood and significant hepatobiliary clearance and intestinal reuptake. Liposomal nanoparticle delivery systems may favorably alter the pharmacokinetic profile and improve tumor targeting of highly lipophilic but otherwise promising cancer imaging tracers, such as the EGFR inhibitor SKI243. SKI243 is therefore an interesting model molecule for incorporation into lipid-based nanoparticles, as it would not only improve their solubility but also increase the circulation time, availability and, potentially, targeting of tumors. In the current study, we compared the pharmacokinetics and tumor targeting of the bare EGFR kinase-targeting radiotracer SKI212243 (SKI243) with that of the same tracer embedded in liposomes. SKI243 and liposomal SKI243 are both taken up by tumor xenografts but liposomal SKI243 remained in the blood longer and consequently exhibited a 3- to 6-fold increase in uptake in the tumor among several other organs.

Graphical Abstracts

Liposomal radiotracer remained in the blood longer and consequently exhibited a 3- to 6 fold increase in uptake in the tumor among several other organs

Biodistribution (%ID/g) of I-124 radiolabeled SKI 243 encapsulated in liposomes and I-131 labeled SKI 243 co-injected in the same animal after 24 h

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I-124 SKI 243 in nude mice bearing H3255 xenografts, Trans axial image A, 2 h; B, 22 h; C 48 h and liposomal formulation of I-SKI 243 in H3255 bearing nude mice D, 2 h; E, 22 h and F, 48 h. Tumor is circled

1. Introduction

In principle, tumor cells can be specifically detected based on characteristic alterations in oncogenes and other key molecular components. Oncogene products can therefore be used as targets for imaging and therapy. The epidermal growth factor receptor (EGFR) is a cell surface receptor over-expressed in several cancers. There are currently three Food and Drug Administration (FDA)-approved EGFR inhibitors available: Iressa, Tarceva and Lapatinib. Positron emission tomography (PET) with EGFR kinase-specific radiotracers could provide a means for non-invasively characterizing EGFR expression and signaling activity in patients' tumors before, during, and after therapy with EGFR inhibitors [1,2,3,4,5].

Our group has developed a number of PET tracers that bind to EGFR, including the iodinated anilinoquinazoline core-based EGFR inhibitor SKI212243 (SKI-243) [6]. In addition to having the same quinazoline core as Iressa and Tarceva, SKI-243 has a high affinity for the sensitive non-small-cell lung cancer (NSCLC) cell line but not the insensitive

cell lines. However, because these PET tracers' high hydrophobicity ($\log P \sim 4$) leads to rapid hepatobiliary clearance and high intestinal uptake, additional formulation steps are required for the optimization of the pharmacokinetics of these imaging agents [7,8,9].

Previously, it has been stated that the hydrophilicity of these drug compounds with a quinazoline core can be increased by adding polyethyleneglycol (PEG) moieties to the compound [8]. However, such structural alterations can lead to decreased binding affinity or decreased cell membrane permeability or both [10].

Liposomal nanotechnology provides a highly versatile platform for exploring several different approaches to improving the tumor targeting of drugs and has been shown to effectively improve selective localization in human tumors *in vivo* of small-molecule drugs such as doxorubicin [11]. Liposomes target tumors spontaneously because of the fenestrated blood vessels of the tumor, and result in enhanced permeability and subsequent drug retention. Liposomes also offer a platform for further active targeting by using multiple targeting agents linked to the liposome core [12,13,14]. Using PEG-coated lipid-based nanoparticles, we sought to improve both the circulation time and solubility of SKI 243, and simultaneously increase its availability to the tumor without adversely affecting its binding to EGFR. Liposomal SKI 243 also works as a great model molecule for an iodinated small hydrophobic molecule, and the data will be useful in the future as a guideline for further studies of a variety of similar molecules that are encapsulated in liposomal systems.

In the current study, we compared the pharmacokinetics and tumor targeting of the bare EGFR kinase-targeting radiotracer SKI212243 (SKI243) with that of the same tracer embedded in liposomes. To our knowledge, the effect of encapsulating a small high-affinity radiotracer in a liposome on the uptake in tumor xenografts has not been evaluated to date. Although it has been stated that the passive targeting of liposomes increases the amount of the drug in tumors, it has not been shown how much of that drug can actually reach its molecular targets. If the amount of radiotracer localizing in the tumor could be increased substantially by using passively-targeted delivery, then the limiting parameter for the binding of the tracer would potentially be the receptor density. Because liposomal nanoparticles can also be used in drug delivery, combining a PET tracer with nanoparticle delivery should not only provide new insights into the relative EGFR expression of the tumor cells, but also important information for the development of drug delivery systems and dosing.

Here we demonstrate that a liposomal nanoparticle system can both improve specific PET-based molecular imaging of EGFR of a lung H3255 cell xenograft that expresses EGFR^{L858R} mutation. This mutation makes the cell line super sensitive to Iressa, the closest chemical derivative of SKI 243 ¹²⁴I-anilinoquinazoline radiotracer. These features of a liposomal nanoparticle system may ultimately lead to improved non-invasive and therapeutically-significant molecular characterization of human lung cancer [15].

2. Materials and methods

Labeling of SKI 243

The synthesis of the anilinoquinazoline-based EGFR inhibitor SKI243 (aka SKI-212243, IPAQ) was initially based on our previously reported procedure [3]. Briefly, [^{131}I]-NaI solution (MDS Nordion or Perkin Elmer) or [^{124}I]-NaI solution (5 mCi in 10 μl in 0.1N NaOH) was added to a solution of 20 μl of Sn-SKI-243 precursor (2.0 mg in 1ml methanol) in a Reacti-Vial. The resulting solution was vortexed for 10 seconds. 15 μl of chloramine-T solution (1 mg/ml) in acetic acid was added to this solution and vortexed. The solution was then heated at 50°C for 30 minutes and allowed to cool to room temperature.

The reaction mixture was diluted with 100 μl of 50% acetonitrile in water and injected into an HPLC. The quenching of the reaction mixture was not needed because the crude reaction mixture was immediately injected into the HPLC (analytical column) for purification.

The product was purified by passing through a C-18, RP HPLC column (Phenomenex Luna 250 $\times$ 4.6 mm) using the gradient of 20-100% B (3-20 min) (A = 0.2% acetic acid; B = acetonitrile) as the eluant with a flow rate of 1 ml/min. The product had a retention time of about 11 minutes and was collected and the solvent removed under reduced pressure. The product was reconstituted in 0.9% saline for further studies. The radiochemical yields calculated from HPLC trace were always >95% with specific activity of 992mCi/micromole

Liposome formulations

Liposomes were prepared by using a lipid mixture consisting 15 mmol/L of DSPC:cholesterol: 3400 PEG-PE. Lipids stored in chloroform were pipetted into a round-bottomed flask, dried under nitrogen and lyophilized for 2 h to remove trace amounts of chloroform. Radiolabeled SKI-243 in PBS was added to the lipids. The lipids were allowed to hydrate for 30 min at 60°C. Extrusion was performed 11 times through a 100-nm polycarbon membrane using a small-volume extruder. Liposomal SKI 243 was purified from the unbound SKI 243 by a PD 10 column, with the liposomes appearing in the void volume.

Physicochemical Properties

Monolayer studies—Lipid monolayers residing on an air-water interface provide a convenient means to assess the lipophilicity of small molecules by monitoring the increase in surface pressure caused by the insertion of the drug of interest into the film [16]. Penetration of the indicated molecules into PC films was measured using circular wells (subphase volume, 400 μl). Surface pressure (π) was monitored with a Wilhelmy wire attached to a microbalance (μ ; TroughS, Kibron Inc., Helsinki, Finland). The lipids were spread on the air-buffer (PBS [pH 7]) interface in chloroform (~ 1 mg/ml) to create different initial surface pressures (π_0), and the resulting monolayer was allowed to equilibrate for 15 min before the addition of the SKI 243 into the subphase. The increment in π from the initial surface pressures (π_0) after the addition of SKI 243 was complete in ~ 20 min, and the difference between π_0 and the value observed after the binding of the peptide into the film was taken as π . All of the measurements were performed at ambient temperature ($\sim 24^\circ\text{C}$).

Specific activity

The size of the liposomal nanoparticles was measured by dynamic light scattering (Zetasizer Malvern). Size can be evaluated before and after PD 10 column purification in order to detect aggregates. The amounts of the liposomes and of the radioactive drug were assessed by centrifugation at 10000 g for 30 min (liposome technology, Gregoriadis). Radioactivity in the supernatant and the pellet was measured by a Wallac gamma counter. The specific activities (in $\mu\text{Ci/mol}$) of the liposomes and the radioactive drug were calculated based on the foregoing measurements.

In Vitro binding studies

Displacement studies were performed with ^{131}I -SKI 243 and H3255 non small cell lung cancer cells and cold SKI243. Briefly, triplicate samples of cells were mixed with $<1\text{ nM}$ of ^{131}I -SKI 243 and increasing amounts of a cold competitor (1 pM to $1\mu\text{M}$). The solutions were shaken on an orbital shaker and after 45 minutes the cells were isolated and washed with ice cold Tris buffered saline using a Brandel cell harvester. All the isolated cell samples were counted and the specific uptake of ^{131}I -SKI 243 determined. These data are plotted against the concentration of the cold competitor to give sigmoidal displacement curves. The IC_{50} values were determined using a one or two site model and a least squares curve fitting routine (Origin, OriginLab Corp, Northampton, MA)

Tumor inoculation

All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committees of Memorial Sloan Kettering Cancer Center and followed National Institutes of Health guidelines for animal welfare. Male athymic nu/nu mice (6-8 wks old, Taconic Farms, NY) were provided with water and maintained on regular diets ad libitum.

Nude mice were injected subcutaneously with an equal volume of Matrigel and cell solution in the flank or shoulder with 3×10^6 A431 or 6×10^6 H3255 cells, respectively. When tumors were 5-8 mm in diameter, the mice were anesthetized with a 2% isofluorine/98% oxygen mixture and injected intravenously. Activity was assayed in a dose calibrator prior to injection, the residual activity was assayed after injection and the actual activity administered was calculated as the difference. The injected dose's volume was less than 300 μl ; its pH was 7-7.9; and its osmolarity was similar to that of blood.

PET imaging

When tumors were 5-10 mm in diameter, mice were anesthetized with a 2% isofluorine/98% oxygen mixture and injected intravenously with ^{124}I labeled SKI 243 ($\sim 100\mu\text{Ci/mouse}$) or liposomal I-124 labeled SKI 243. PET imaging was performed with a Focus 120 micro PET.

Biodistribution of liposomal SKI 243 in A431 and H3255 cell lines—Different formulations of SKI-243 ($\sim 20\mu\text{Ci/mouse}$), ^{131}I -SKI 243 or liposomal I-131/I-124 labeled SKI 243 were prepared and used. Liposomal In-111 was coupled to liposomes by using DOTA-PE. Doses were injected into the mice via the tail vein (IV), and animals were sacrificed by CO_2 asphyxiation at 1 h, 4 h, and 24 h post-injection, with three animals per

time point. Tumor and organs, including blood, heart, lung, liver, spleen, kidney, small intestine, large intestine, stomach, muscle, femur, brain and tail, were removed, washed in PBS to remove adherent blood, blotted dry, weighed and radioassayed for I124 and/or I131 in a scintillation well counter calibrated for the respective isotopes. Net count rates were converted to the percentage of the injected doses per gram (%ID/gm), and corrected for radioactive decay at the time of injection.

Results

Radiolabelled SKI-243 was biologically active and the displacement binding curve was monophasic, suggesting a single binding interaction. The IC_{50} of SKI243 for inhibiting ^{131}I -SKI243 was 7.7 ± 3.8 nM (n=3) indicating a K_d of around 8 nM.

SKI-243 is an amphiphilic compound in a pH of 7.5. It is incorporated into the PC monolayer at surface pressures up to 22 mN/m. Encapsulation of SKI-243 in PC:Cholesterol and PEG-PE liposomes was found to be facile, with yields of the encapsulated tracer after the PD 10 column purification of 10-15% (Fig. 1a) and a release rate from the liposomes of 2.5%/h.

Therefore, liposomal preparations were formulated immediately before experiments. After encapsulation, liposomes were approximately 100 nm in size (Fig. 1b). The biodistribution of SKI-243 and liposomal SKI-243 in mice bearing H3255 tumor xenografts at 1 h, 4 h, and 24 h post-injections are shown in Figure 2, and the corresponding tumor-to-organ activity concentration ratios are shown in Figure 3. The blood clearance and biodistribution of SKI-243 and liposomal SKI-243 are clearly different; the blood circulation time of SKI-243 increased because of the liposomal delivery system. The biodistribution of labeled SKI 243 and liposomal SKI 243 was determined, and shown in Figure 2.

The absolute uptake varies according to status of the tumor and the quality of the injection but the ratio between the liposomal delivery and bare drug was constant in all major organs. Typical experiment is shown in the fig 2. The highest uptake of SKI 243 and liposomal SKI 243 in H3255 tumors appeared 1 h post-injection (1.05%ID/g and 4.4%ID/g, respectively); these two compounds in normal muscle tissues were lower (0.24%ID/g and 1.2%ID/g, respectively). The liposomal delivery increased tumor uptake at 1 h nearly 4-fold, but spleen and liver uptake increased 60- and 10-fold, respectively.

The tumor-to-muscle ratios increased over time, reaching 5 for bare SKI-243 and over 10 for the liposomal formulation at the later time points. The tumor-to-blood ratio likewise increased over time and, at 24 h, was 2 for bare SKI-243, and over 4 for liposomal SKI-243 (Fig. 3c).

PET imaging showed that H3255 and A431 flank tumor xenografts in mice could be visualized. This was confirmed by SPECT imaging of ^{131}I -SKI-243 (data not shown). Dynamic images of SKI-243 and liposomal SKI-243 in EGFR-expressing H3255 tumor xenografts showed tumor uptake in the four hours (Fig. 4a and Fig. 4b) with the tumors still clearly visible at 48 h. The tumor to back ground ratio goes up with liposomal SKI 243 as

bare SKI 243 but the overall signal of bare SKI 243 is so low in 48h that meaningful analysis of tumor to background is difficult to perform.

There is a clear difference in the images between liposomal and non-liposomal formulations, and specifically, at the later time points, the tumor is more clearly visualized with the liposomal formulation. The biodistribution of co-injected I-124-SKI 243 encapsulated in liposomes and bare I-124-SKI 243 at all three time points confirms these statistically relevant differences between the liposomal and **non-liposomal formulations** (Fig. 5, supplemental fig 1).

The blood clearance is substantially slower in regard to liposomal delivery. There is also a 5 fold difference in the tumor uptake at 1 h time point, over the liposomal formulation versus bare radiotracer but increased uptake of the former in the liver and spleen. Liposome with In-111, co-administered with liposomal I-124 labeled SKI 243, showed that there was a different gastrointestinal secretion pattern between In-111 DOTA PEG-PE labeled liposome and encapsulated SKI 243 at the 24 h time point this points to the direction that the SKI 243 is able to leak out from the liposomes and bind to the EGFR target

Discussion

SKI 243 is a lipophilic compound that readily incorporates into the PC lipid monolayer at a surface pressure of 22 mN/m. This means that it is highly likely that it adheres to a lipid bilayer and thus across cell membranes [17]. In this study, liposomal formulation prolonged the blood circulation time and tumor accumulation of SKI 243 in both H3255 and A431 mouse xenograft models observed by PET. Previously [6], it was shown that SKI 243 targets to EGFR expressing A431 xenograft tumors in mice and rat models. There has also been region of interest analysis based estimation of %ID/g up to one hour. Our results with A431 tumor uptake are roughly similar in PET. Accumulation of liposomal SKI 243 in the H3255 tumors was likely due to the effect of the passive targeting of solid tumors by the liposomes (ERP). Because the drug is amphiphilic, it will eventually leak out from the liposomes, and the resulting free imaging agent may therefore interact with cells. Although a more stable liposome would possibly increase drug accumulation in the extracellular compartment of the tumor because of the generally greater permeability of tumor blood vessels, the slower drug release rate would hinder the binding of the SKI-243 to the EGFR. We found that the liposomal formulation increased tumor uptake some 3- to 6-fold. The tumor-to-muscle ratio at 24 h increased from 4 to 10 with liposomal formulations, and the tumor-to-blood ratio increased from 2 to 4. Co-administration of radiolabeled forms of the liposomal formulation and the bare drug clearly resulted in slower blood clearance and higher tumor uptake of the liposomal formulation, but also caused increased uptake in the liver and the spleen. In addition, the study showed apparent differences in tumor uptakes related to the size and status of the tumor. However liposomal formulations were able to deliver significantly more SKI 243 to the tumor site than the bare formulation.

High uptakes of the stomach, small intestines and large intestines hint that there is an amount of free iodine during the 1 h time point. This could be due to the deiodination of the SKI 243. There is clear pattern of secretion of the radioactivity over the time course, from

the stomach to the small intestines, and then to the large intestines. With liposomal formulation, this pattern is similar but slower, possibly because of the protection of liposomal SKI 243 from metabolisms like deiodinases. The In-111 labeled liposome vs. SKI 243 experiment reveals that the biodistribution of the radiolabeled lipid and the iodinated drug is different in the first hour and increasingly so after 24 hours. Also, encapsulated drug and lipid core have different biodistribution after 24 hours. The lipid core of the liposomes goes mainly to the liver and to the spleen, and the rest of the drug is secreted through the liver to the hepatobiliary system. This is reasonable considering the leakage of the liposomes is 2,5%/h in test tube. All of this supports the idea that the increased tumor uptake is specific in nature and not solely due to the EPR effect without active binding.

Improvements to both the specificity of the quinazoline inhibitor and the liposomal formulation can still be made: the first improvement would be to include a targeting agent or internalizing agent or both with the liposome; the second improvement would be to change the radioisotope and the labeling method to something more stable such as F-18 or Zr-96. The biodistribution of EGFR-binding drugs can thus be altered by liposomal carriers, including enhancement of passive tumor localization. Also packing the the radiotracer opens a possibility to image guided delivery system development. Liposomal formulations of SKI243 and lipophilic tyrosine kinase inhibitors appear promising as a means of enhancing the tumor targeting of such drugs, with potential wide applicability to poorly soluble or cell membrane- and blood-brain barrier-impermeable agents.

Mutated EGFR is overexpressed in about 10% of lung cancers, and therefore liposomal SKI 243 or other quinazoline core imaging agents can be used to select patients that are responsive to EGFR inhibition-based therapy and potentially can enhance targeting of such therapeutic agents to responsive tumors [18]. At the same time, monitoring the effectiveness of such therapy may be improved with liposomal formulations of radiolabeled inhibitors such as SKI 243.

Liposomal or micellar formulations are often used to increase the solubility of drugs. However, if nanoparticles or other advanced drug delivery methods are used to increase the solubility of drugs, it is important to understand the effect of the delivery system on the overall biodistribution of the drug [19,20]. Liposomes, micelles and other nanoparticles can be used as carriers of imaging tracers, and imaging results can then be used for the development and optimization of drug delivery systems for therapy [20,21,22,23]. Liposomal SKI 243 can be used as a model molecule for an iodinated small hydrophobic molecule, and these data can be applied to the great variety of similar molecules that are encapsulated in the liposomal systems. There are also several promising novel quinazolines that are in developmental or clinical trials [24,25,26]. Using liposomes will likely increase both the solubility of these hydrophobic drugs and the circulation time in the body and and also achieve better tumor targeting. There are already reports that the pharmacology and pharmacokinetics of EGFR inhibitors (erlotinib, gefitinib) affect the clinical efficacy of the drugs [27] and improving them has being exactly what we have shown in this paper. Leakage of the hydrophobic and amphiphilic compounds from the liposomes might be a problem during storage. However, if the liposomes are constructed immediately prior to use this problem can be solved or additional formulation steps can be made to improve the shelf

life. On the other hand, small and steady leakage can be advantageous in drug delivery stand point, and in situ preparation of the liposomes is relatively simple, even in a hospital environment. The applications of such platforms as vehicles for targeted delivery can be expanded by the coupling of antibodies, peptides and specific small molecules to the liposome surface. In addition, alterations of liposomal formulations may favorably alter their pharmacokinetic profiles. Some liposomal products are already FDA-approved for IV formulations; this should facilitate the translation of nano-delivery systems to clinical trials. Such formulations have potentially wide applicability in personalized nanomedicine.

Liposomal SKI 243 as such may not be feasible enough to be used directly in a clinical setting. However, liposomal delivery combined with other drug compounds with a quinazoline core might open promising alternatives for clinical imaging and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

c

Fig. 1.

Purification and quality control of liposomal I-124 labeled SKI 243. a, Purification liposomes by size exclusion PD 10 column chromatography; 1.3 ml fractions. b, Size distribution of SKI 243 liposomes by Zeta sizer. c, Displacement assay with H3255 cell lines that were I-124 labeled SKI-243 is displaced with nonradioactive SKI-243.

A

B

c

Figure 2.
Biodistribution of liposomal I-124 labeled SKI 243 in mouse bearing H3255 tumor xenografts at 1 h (A), 4 h (B), and 24 h (C) post-injection.

A

B

c

Figure 3.

Tumor-to-organ activity concentration ratios at 1 h (a), 4 h (b), and 24 h (c) post-injection.

Fig 4.

A. Transaxial microPET images of I-124-labeled SKI 243 in nude mice bearing A431 xenografts at 2, 22, 48, and 63 h post-injection. Upper row: “bare” (non-liposomal) formulation of I-124-SKI 243. Bottom row: Liposomal formulation of I-124-SKI 243. The tumor xenograft is identified by the arrows. The images as displayed were smoothed and individually scaled to optimize the visualization of the tumor-to-background contrast.

B. Transaxial microPET images of I-124-labeled SKI 243 in nude mice bearing H3255 xenografts at 2, 22, and 48 h post-injection. Upper row: “bare” (non-liposomal) formulation of I-124-SKI 243. Bottom row: Liposomal formulation of I-124-SKI 243. The tumor xenograft is identified by the circles. The images as displayed were smoothed and individually scaled to optimize the visualization of the tumor-to-background contrast.

C. Tumor to background ratio of liposomal SKI 243 and bare SKI 243 at 48h time point in H3255 cell line

a

B

Fig. 5.

a. Biodistribution (%ID/g) of I-124 radiolabeled SKI 243 encapsulated in liposomes and free I-131 labeled SKI 243 co-injected in the same animal after 1, 4 and 24 h.

B. Organ to muscle ratios of I-124 radiolabeled SKI 243 encapsulated in liposomes and In-111 DTPA-PE which is localized on the liposome core co-injected in the same animal and analyzed by biodistribution at 24 h timepoint.

Schematic Image 1.

The Tarceva Iressa, and Lapatinib have a quinazoline core as a common structural feature. We have developed an iodinated analog called SKI-212243, that is based on this chemical motif, with the presence of endogeneous iodine to allow incorporation of the positron emitter I-124 for PET. Below are the structural image of SKI 243 and the structural images of other clinically used drug compounds with a quinazoline core: Erlotinib, Gefitinib, and Tykerb.