厚生労働科学研究費補助金

医療機器開発推進（医療機器開発
（ナノテクノロジー等）総合推進）
研究事業

腫瘍血管内皮細胞への薬物送達システムによる耐性癌の化学療法と臨床応用へ向けた製剤化

平成２４年度　総括研究報告書

研究代表者　畠山　浩人

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目　　次

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腫瘍血管内皮細胞への薬物送達システムによる耐性癌の化学療法と臨床応用へ向けた製剤化

研究代表者　畠山　浩人　北海道大学大学院薬学研究院　特任助教

研究要旨

がん細胞自身ではなく血管新生を阻害することは耐性癌治療の有用な手段であり、薬物送達システム(DDS)による腫瘍血管内皮選択的な抗癌剤の送達は、効果の向上と副作用の軽減に有効である。申請者は標的化リガンドと細胞膜透過性ペプチドを組み合わせたdual-ligandリポソームにより腫瘍血管へ選択的な薬物送達と耐性癌治療に成功している。従来治療効果に乏しい癌種でもDDSで血管に薬物を送達し抗腫瘍効果が示されれば、既存の抗癌剤の適応拡大とライフサイクルの延長など医薬品産業の競争力を下支えする基盤技術として貢献が期待される。またポソーム製剤の開発を通じて医薬品レギュレーション行政へ貢献が期待される。本事業では、dual-ligandリポソームを用いた耐性癌化学療法を最終目的として、適応癌種拡大と非臨床試験へむけた製剤化の検討を行った。

適応癌種の拡大では腎細胞癌だけではなく、17種の癌細胞からドキソルビシンに強い耐性を示す肺癌、乳癌、卵巣癌、膵癌の４種を同定した。また新たに２種類標的分子（IntegrinとVEGF-R2）のリガンド分子を搭載した新規dual-ligandリポソームの構築に成功した。一方で、製剤化への取り組みについては、当初計画を前倒ししGMP製造にむけたdual-ligadリポソームの大量製造法の検討に着手し、300 nmのdual-ligandリポソームの至適調製条件を見出すことに成功した。また従来1 mL程度であった製造スケールを10 mLへ向上させた。

A. 研究目的

薬剤耐性は癌の化学療法を困難にする深刻な問題である。主な原因はがん細胞が薬剤に抵抗性を示すP糖タンパク質などの分子を発現するようになることが挙げられる。近年、がん細胞自身ではなく、がん細胞に栄養や酸素を供給する腫瘍血管を標的とした血管新生阻害療法が注目されており、薬剤耐性癌にも有効であることが報告されている。しかし、一方では消化管出血など重篤な副作用も報告されている。これは、がん組織以外の正常組織の血管においても血管新生阻害剤が薬効が発揮されてしまうことが原因と考えられている。従って、疾患部位であるがん組織の腫瘍血管に選択的に薬物を送達可能な、薬物送達システム（Drug Delivery System: DDS）

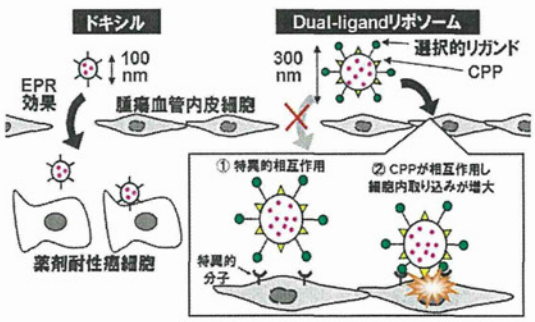

Figure 1. Dual-ligandリポソームの概念図

は治療効果の増強と副作用の軽減に有用な手段の一つと考えられる。

これまでに我々は、腫瘍血管内皮細胞に選択的に薬物を送達可能な腫瘍血管内皮標的化dual-ligandリポソームの構築に成功している（PCT/JP2011/053963）。Dual-ligand

リポソームは、腫瘍血管に特異的に発現している標的分子を認識する選択的リガンドと、細胞内取り込みを飛躍的に向上させる細胞膜透過性ペプチド（Cell penetrating peptide: CPP）が修飾されている。また、腫瘍血管への親和性を向上させるためリポソームの直径を300nmに制御している（Fig. 1）。既に臨床において承認されているドキソルビシン封入リポソーム製剤Doxil®ではまったく効果のない薬剤耐性腎細胞癌OSRC-2担癌モデルにおいて、ドキソルビシン封入dual-ligandリポソームは腫瘍血管を破壊し、腫瘍増殖を有意に抑え抗腫瘍効果を示した。ドキソルビシン投与量も通常よりも低く抑えられ、体重減少や肝毒性などの副作用は見られず安全性も高いことが示されている。本Dual-ligandリポソームについて特許出願を行った際、特許庁からも「すべての発明に特許性あり」（PCT/JP2011/053963）とコメントがあり、dual-ligandリポソームは新規性や独創性の高い送達システムと考えている。

本研究の成果が実用化されると、既存の抗癌剤では効果に乏しい癌種に対して、血管を標的とすることで既存の抗癌剤でも癌治療が可能になり抗がん剤の新たな適応の拡大が見込めるため、医薬品のライフサイクルの延長が見込まれ、医薬品産業の競争力を下支えする基盤技術として貢献が期待される。またポソーム製剤の開発を通じて医薬品医療機器総合機構「医薬品・医療機器薬事戦略相談」を活用し、ナノテクノロジーを基盤とする医薬において必要とされる試験や規格化すべき物性などの検討を通じて、医薬品レギュレーション行政へ貢献できると考えている。

以上より本研究事業では、腫瘍血管へ抗癌剤を送達し薬剤耐性癌を治療可能なdual-ligandリポソームの臨床への応用を最終目的として、適応可能な薬剤耐性癌種の拡大に向けた新規dual-ligandリポソーム開発と、非臨床試験に供するGMP基準の製剤化に向けたdual-ligandリポソームの大量調製法の確立を目的として研究を遂行した。

B. 研究方法

① 新規dual-ligandリポソームの開発腫瘍血管内皮標的性の評価

研究開始時、dual-ligandリポソームは、腫瘍血管内皮細胞に高発現しているアミノペプチダーゼN（CD13）を標的として標的リガンドにNGRモチーフを含むペプチドを、CPPとしてステアリル化テトラアルギニンを修飾したシステムであった。癌種、腫瘍血管の多様性を考慮し、別の標的分子を標的化可能な新規dual-ligandリポソームの開発を行った。

Dual-ligandリポソームは単純水和法で調製した。Egg phosphatidylcholine (EPC)、cholesterol (Chol)、polyethylenglycol (PEG)脂質、標的リガンド修飾PEG脂質、およびステアリル化オリゴアルギニンを、EPC:Cholが7:3、その他が任意の含量となるように有機溶媒に溶解させ、減圧下で溶媒を留去し脂質薄膜を調製した。任意の緩衝液で脂質薄膜を水和し、撹拌することで脂質薄膜を剥離させ、800 nmのポアサイズのポリカーボネート膜を用いて整粒した。粒子径は動的光散乱法（Zetasizer Nano ZS ZEN3600、MALVERN社）によって測定した。

新生血管に高発現しているインテグリン（Integrin）αvβ3と血管内皮増殖因子（vascular endothelial growth factor: VEGF）レセプター2（VEGF-R2）に対して高親和性を示すペプチド配列としてRGDモチーフペプチドとATWLモチーフペプチドの選定にすでに成功しているため、インテグリンαvβ3とVEG-R2分子を標的候補とした。標的化リガンドペプチドをdual-ligandリポソームのPEG（平均分子量2000）鎖の先端に修飾し、CPPリガンドとしてステアリル化オリゴアルギニン（STR-RX）をリポソーム表面に修飾した。これらのリポソームの脂質組成にローダミン脂質を0.1%加えることで蛍光標識を行い、ヒト臍帯静脈

由来HUVEC、さらには担癌マウスの腫瘍組織から単離された腫瘍血管内皮細胞（Tumor endothelial cells: TECs）への取り込み量について、リポソーム添加後2時間インキュベーションした後、観察直前に細胞核をHoechst33324で染色後に、Nikon A1（対物レンズ×40）で観察し画像を取得した。また細胞取り込み量について、細胞を回収し溶解させた後、遠心分離にて得られた上清の蛍光強度を測定することでも強化した。これらの測定結果をもとにリガンド修飾条件などリポソーム組成の最適処方を決定した。

また、ドキソルビシン耐性癌である腎細胞癌由来OSRC-2をBALB/cヌードマウス（雄性、4－5週齢、日本クレア）の背部皮下に移植後、腫瘍径が100mm^{3}になった時点で、ドキソルビシン量として1~2mg/kgの投与量で、担癌マウスの尾静脈より投与し、経時的に腫瘍体積を測定し、既存のリポソーム製剤であるDoxilと比較することで抗腫瘍効果を評価した。

② 適応癌種拡大にむけた耐性癌種の同定とin vivoモデル確立

研究開始時、Dual-ligandリポソームの有効性が確認されている癌種は腎細胞がんOSRC-2のみであったため、適応癌種の拡大を目的として市場の大きい肝癌や肺癌、難治性の膵癌などを中心として、17種類の癌細胞を用い、ドキソルビシンへ耐性の高い癌種の選定を行った。各癌細胞に対して、培地に各濃度となるようにドキソルビシンを添加後、8時間インキュベートした。ドキソルビシンの除去と洗浄後にさらに16時間インキュベートし、細胞生存率をcell counting kit-8（Dojindo）を用いて評価し、薬剤反応曲線から算出されたEC50から、ドキソルビシン耐性癌種を選定した。

③ GMP基準の製剤化に向けたdual-ligandリポソームの大量調製法の確立

当初二年目（平成25年度）に予定していた大量調製法の確立にむけた検討を、計画を前倒しして、平成24年度から開始した。研究開始時、dual-ligandリポソームは有機溶媒に脂質を溶解し、減圧下で溶媒を留去することで脂質薄膜を調製し、その後緩衝液で水和させ調製していた（単純水和法）。しかし、単純水和法は操作が煩雑で試験管一本あたりのリポソーム調製量は500 μL~1 mLと少ないため動物実験では何本もの試験管で調製するため、試験管毎に物性が異なる場合があり、ロット差も問題となるため大量調製は困難であった。

そこで、非臨床試験を考慮に入れ、リポソーム製剤のGMP基準での製剤化に向けた大量調製法の確立を目的に、バイオメッドコア社のマイクロインラインアセンブラー技術によるGMP対応可能なリポソーム連続製造装置を用い大量調製法の確立にむけた基礎検討を行った。インラインアセンブラーによる検討の前段階として、バイオメッドコア社では条件検討をバッチ法により行っており、本事業でも初めにバッチ法による条件探索を行った。Dual-ligandリポソームの粒子径が300 nmとなる調製条件について、溶媒濃度、加温温度、脂質濃度などのパラメーターを様々ン変化させ、最適条件を探索した。

なお、本研究事業は動物実験を実施するため、北海道大学が定める「動物実験に関する規定」に基づく承認を取得済みであり、必要最低限の個体数や癌治療実験におけるエンドポイントの設定など、動物愛護上の配慮を行い遂行した。

C. 研究結果

① 新規dual-ligandリポソームの開発腫瘍血管内皮標的性の評価

RGDモチーフを含むインテグリン標的化ペプチドおよびSTR-R8を用いたdual-ligandリポソームを調製した。HUVECおよびTECを用いた細胞取り込み試験の結果、RGD結合PEG脂質とSTR-R8はそれぞれ総脂質量に対

して5%および2%が最適であることが明らかとなった。そこで、これらの処方で調製されたドキソルビシン封入dual-ligandリポソームを、ドキソルビシン量で1.5 mg/kgで腎細胞癌OSRC-2皮下移植担癌マウスの尾静脈より3回投与した。リポソーム製剤Doxilではまったく効果が示されなかったが、dual-ligandリポソーム投与群では有意に腫瘍増殖が抑制された（Fig. 2）。以上の結果より、インテグリン標的型dual-ligandリポソームはin vivoでも機能し薬剤耐性癌治療に使用可能であった（論文投稿準備中）。

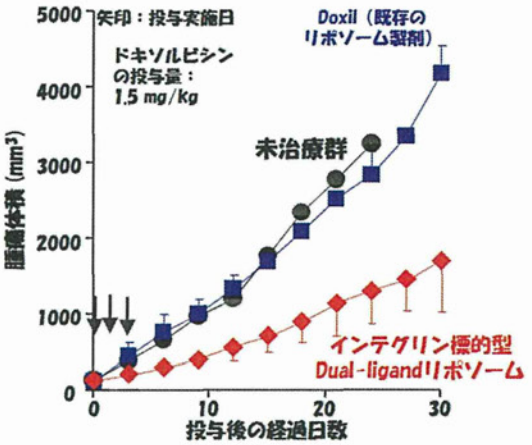

Figure 2. インテグリン標的型dual-ligandリポソームのドキソルビシン耐性癌における抗腫瘍効果

一方、VEGF-R2標的化では、ATWLモチーフを含むペプチドおよびSTR-R8を用いたdual-ligandリポソームを調製した。脂質膜を蛍光標識し、VEGF-R2強陽性HUVECやTECを用いて、細胞取り込みを共焦点レーザー顕微鏡で観察した。その結果、7.5%のATWL標的化ペプチドもしくは2%のSTR-R6どちらかの修飾では取り込みの増加は見られなかった。一方、両方を同時に修飾したdual-ligandリポソームではHUVEC細胞内にリポソームを示す強い蛍光シグナルが認められ、細胞取り込みが飛躍的に上昇することを確認した（Fig. 3）。以上の結果より、標的リガンドにより修飾量に最適値が異なるり、標的リガンド毎に最適処方を見出す必要があることが示唆された。今後はin vivo担癌マウスを用い、腫瘍血管内皮への標的性を組織の共焦点レーザー顕微鏡観察や放射背同位体標識による体内動態の評価によって確認し、ドキソルビシンなど抗癌剤を封入し抗腫瘍効果や延命効果を指標に機能評価を進める。

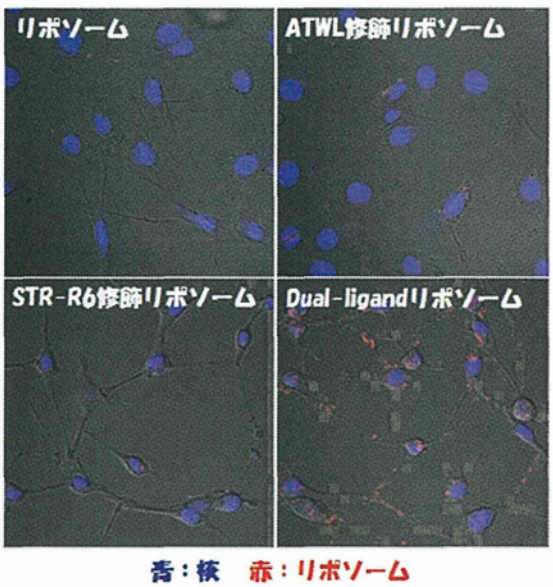

Figure 3. VEGF-R2 標的型 dual-ligand リポソームの腫瘍血管内皮細胞への取り込み

② 適応癌種拡大にむけた耐性癌種の同定とin vivoモデル確立

研究開始時には耐性癌モデルは腎細胞癌のみであったため、17種類のヒト癌細胞に対して、ドキソルビシン感受性を検討した。その結果、癌種によってドキソルビシン感受性は大きく異なり、EC50には1000倍以上のダイバーシティが存在することが明らかとなった。また、OSRC-2と同程度にEC50が高くドキソルビシンに耐性を示す癌種とし、肺癌（H69AR）、乳癌（MDA-MB-231）、膵癌（PANC-1）、卵巣癌（SKOV-3）を見出すことに成功した（Fig. 4）。今後は、この4種の癌細胞を用いてin vivo担癌モデルを作成し、従来のものや①で構築された

新規dual-ligandリポソームを用い、癌種によって最適化リガンド処方を決定する。

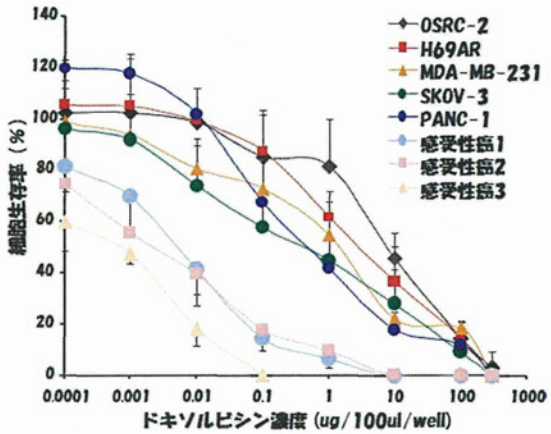

Figure 4. ドキソルビシンに対する感受性試験

③ GMP基準の製剤化に向けたdual-ligandリポソームの大量調製法の確立

閉鎖流路による連続製造を行う前段階としてバッチ製造による条件検討を行った。10～50 mLのガラス瓶にて脂質を溶解させた有機溶媒と水溶性緩衝液を混合させ調製する。その際、緩衝液の濃度やpH、有機溶媒と緩衝液の容積比、脂質濃度、加温温度など様々なパラメーターが、リポソームの物性に影響をあたえるため、300 nmのリポソームが調製可能な至適条件を探索した。その結果、至適溶媒濃度や脂質濃度を見出し、非常に均一で単分散の分布をもつ直径約300 nmのリポソームを調製することに成功した（Fig. 5）。

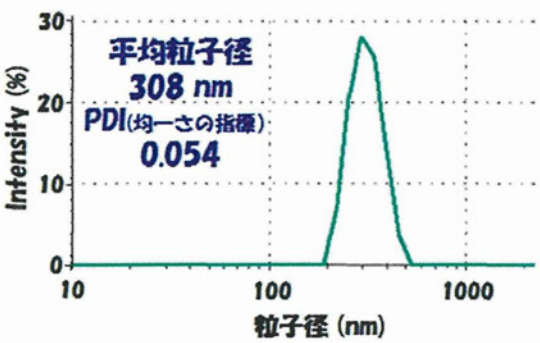

Figure 5. 動的光散乱法により測定したdual-ligandリポソームの粒度分布

従来の単純水和法ではdual-ligandの調製スケールが1 mL/hr程度であったが、バッチ製造の段階で10 mL/hr以上と、研究開始時より10倍程度調製スケールを向上させることに成功した。今後は閉鎖系流路（Fig. 6）を用いた大量製造を検討することで、製造スケールを100mL/hr、当初の100倍以上とすることが見込まれる。

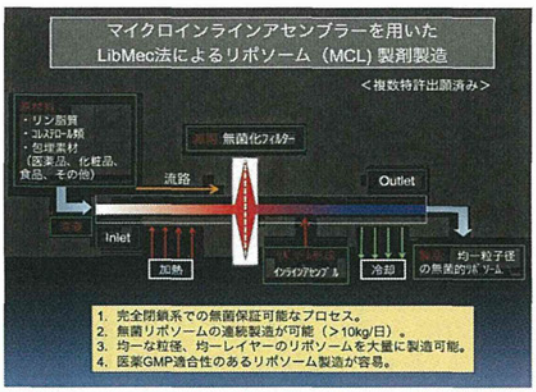

Figure 6. マイクロインラインアセンブラーによるリポソーム製造法の概要

D. 考察

耐性癌種の同定では、4種類のドキソルビシン耐性癌を見出すことに成功した。一方で、ドキソルビシンの細胞内取り込み量を測定したところ、同じ耐性を示す癌細胞でも、その量は大きく異なっていることが明らかとなった。ドキソルビシン耐性への関与がよく知られているP糖タンパク質（Pgp）は、細胞内のドキソルビシンの細胞外への排泄を促進する。H69ARやSKOV-3は細胞内ドキソルビシン量が低く、Pgpが耐性に大きく関与していることが示唆された。一方で、OSRC-2やMDA-MB-231は細胞内ドキソルビシン量が他の癌種と比較して高いにも関わらず耐性を示しており、おそらくドキソルビシン耐性におけるPgpの寄与は小さく、異なる機構によってドキソルビシンに対して耐性を示していると考えており、詳細

について現在検討中である。

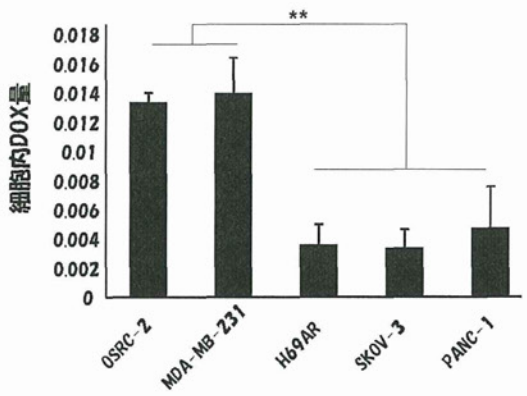

Figure 7. 耐性癌のドキソルビシン取り込み量

Dual-ligandリポソームは腫瘍血管との親和性を向上させる目的で直径を300 nmに制御している。これは一般的に用いられているリポソームの粒子径100 nmと比較して大きい。直径が3倍大きいことから、表面積は約9倍広いため、腫瘍血管との接触面積が増大することで、リポソームの腫瘍血管との親和性が増大している。また300 nmのリポソームの一粒子当たりに封入可能な薬物量は100 nmのものと比較して20～30倍多く、一粒子が送達する薬物量も増大するため、薬効の増強などが、粒子径が大きい場合の利点として考えられる。一方で、粒子径が大きいためdual-ligandリポソームは通常の100 nmのリポソームと比較して肝臓や脾臓といった細網内皮系により認識されやすくなるなど体内動態においてやや不利な点もあるが、肝臓・脾臓への集積による毒性等は観察されていなかった。

粒子径が大きいことで最も大きく影響を受けると考えられるのは製剤化における滅菌の問題であることが予想される。滅菌に用いられるフィルターのサイズは通常200 nmであり、300 nmのdual-ligandリポソームはフィルター滅菌が不可能である。しかし、本事業において製剤化において用いるバイオメッドコア社の閉鎖系の連続流路マイクロインラインアセンブラー技術は、リポソームの構成脂質をフィルターで滅菌し閉鎖流路内に流し込み閉鎖流路内で300 nmのdual-ligandリポソームが製造可能であるため、滅菌に関する問題を解決することが可能となる（Fig.6）。またこの装置は運転時間分、連続してリポソームを製造可能で、装置を並列で連結することで相加的に製造速度を上昇することができる。従って、最終的には調製スケールの大幅な向上が期待され、非臨床試験における製造スケールとしても問題がないと考えられる。

E. 結論

新たなドキソルビシン耐性癌種を４種類見出すことに成功した。今後はin vivoモデルの作出とdual-ligandリポソームを用いた抗腫瘍効果試験を行う。またdual-ligandリポソームの標的分子―リガンドについて２種類追加することに成功した。今後は、先に見出した耐性癌モデルを含むin vivo担癌モデルを用い癌種によるリガンドの最適化と、適応癌種の拡大を行う。

製剤化研究では、当初の予定を前倒ししバイオメッドコア社と連携しGMP製造にむけた大量調製法の条件検討により、300nmのリポソームの調製条件を見出すことに成功し、調製スケールを10倍程度向上させることに成功した。以上より当初の予定を概ね達成した。

F. 健康危険情報

該当なし

G. 研究発表

1. 論文発表

1) Takara K*, Hatakeyama H*, Kibria G, Ohga N, Hida K, Harashima H. Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy. *J. Control. Release*, 161(1): 225-232 (2012) *Equally contribution

2. 学会発表

1) 畠山浩人、高良和宏、キブリアゴラム、大賀則孝、樋田京子、原島秀吉. 腫瘍血管内皮を標的としたDual-ligandリポソームの開発. 日本薬剤学会第27年会. 2012年6月26日. 神戸（神戸国際会議場、兵庫県）（口頭発表）
2) 畠山浩人、原島秀吉. 薬物をがん細胞・血管内皮細胞へ送り分けるDDSの開発. 第28回日本DDS学会学術集会. 2012年7月5日. 札幌（札幌コンベンションセンター）（招待講演）
3) Hatakeyama H, Takara K, Kibria G, Ohga N, Hida K, Harashima H. Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy. 39th Annual Meeting & Exposition of the Controlled Release Society. 2012年7月15-18日. Québec City, Canada. Québec des congrés de Québec.（口頭発表）
4) Kibria G, Hatakeyama H, Ohga N, Hida K, Harashima H. Tumor Vasculature Targeted Size Controlled PEGylated Liposomal Delivery System to Treat Chemotherapy Resistant Renal Cell Carcinoma. 39th Annual Meeting & Exposition of the Controlled Release Society. 2012年7月15-18日. Québec City, Canada. Québec des congrés de Québec（ポスター発表）

H. 知的財産権の出願・登録状況

該当なし

研究成果の刊行に関する一覧表

書籍
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Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy

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ABSTRACT

Anti-angiogenic therapy is a potential chemotherapeutic strategy for the treatment of drug resistant cancers. However, a method for delivering such drugs to tumor endothelial cells remains to be a major impediment to the success of anti-angiogenesis therapy. We designed liposomes (LPs) with controlled diameter of around 300 nm, and modified them with a specific ligand and a cell penetrating peptide (CPP) (a dual-ligand LP) for targeting CD13-expressing neovasculature in a renal cell carcinoma (RCC). We modified the LPs with an NGR motif peptide on the top of poly(ethylene glycol) and tetra-arginine (R4) on the surface of the liposome membrane as a specific and CPP ligand, respectively. The large size prevented extravasation of the dual-ligand LP, which allowed it to associate with target vasculature. While a single modification with either the specific or CPP ligand showed no increase in targetability, the dual-ligand enhanced the amount of delivered liposomes after systemic administration to OS-RC-2 xenograft mice. The anti-tumor activity of a dual-ligand LP encapsulating doxorubicin was evaluated and the results were compared with Doxil®, which is clinically used to target tumor cells. Even though Doxil showed no anti-tumor activity, the dual-ligand LP suppressed tumor growth because the disruption of tumor vessels was efficiently induced. The comparison showed that tumor endothelial cells (TECs) were more sensitive to doxorubicin by 2 orders than RCC tumor cells, and the disruption of tumor vessels was efficiently induced. Collectively, the dual-ligand LP is promising carrier for the treatment of drug resistant RCC via the disruption of TECs.

1. Introduction

Renal cancer patients, in which most frequent histology is renal cell carcinoma (RCC), typically respond poorly to chemotherapy [1,2]. This poor or complete lack of response to chemotherapy in RCC can be mainly attributed to acquired drug resistance, including up-regulated P-glycoprotein (P-gp) which functions as an efflux pump for chemotherapeutic drugs [2]. Although interleukin (IL)-2 or interferon (IFN)-α based immunotherapy is approved for use, RCC is also resistant to this type of chemotherapy [3]. As a result, the resistance of cancer cells to chemotherapeutic treatment remains a major obstacle to the successful treatment of kidney cancer. Recently, new classes of drugs, sunitinib, sorafenib or bevacizumab, which target specific molecules that are related to the angiogenesis process, such as vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs) have been approved for the treatment of RCC [3,4]. Although RCC patients suffer from side effects, the new class drugs appear to have improved clinical benefits [3,4]. This suggests that anti-angiogenic therapy has promise for the treatment of RCC. Further increases in therapeutic activity could be achieved by targeting the neovasculature with nanomedicines that contain ligands that are selective for a specific target.

Endothelial cells in angiogenic vessels express several proteins that are absent or barely detectable in established blood vessels, including αv integrins, VEGFRs, and other types of membrane molecules, such as aminopeptidase N (CD13) [5]. It has been reported that ligand based liposomes that contain RGD or NGR motif peptides that are capable of targeting the neovasculatures can be used to deliver chemotherapeutic drugs [6–9]. The targeted liposomes showed efficient chemotherapeutic activity, particularly when the targeting was via internalizing ligands that facilitate the delivery of the therapeutic contents to an intracellular site of action via the endosome/lysosome pathway. However, because of the limited number of receptors and the recycling of endocytosis, receptor mediated endocytosis is a saturated pathway, which restricts the amount of liposomes that are available for cellular uptake and greatly decreases the magnitude of the pharmacological effect of such preparations.

To overcome this saturated pathway and to obtain further therapeutic efficacy, we developed a dual-ligand based poly(ethylene glycol) (PEG)-liposome (dual-ligand LP). The liposome was modified

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with a target ligand on the terminus of the PEG and a cell penetrating peptide (CPP) was attached to liposome surface [10,11]. Because it is masked by PEG, the CPP is not functional and opsonin-free in the systemic circulation. The recognition of target cells mediated by target ligands and subsequent cellular association permit the CPPs to allow the liposomes to be rapidly internalized by target cells, due to the close proximity of the liposomes to the surface of the target cells. As a result, the cellular uptake and the enhanced activity of the cargo of the dual-ligand LP are vastly superior compared to a liposome mono-modified with a specific ligand.

In the present study, we describe a novel therapy for RCC as a drug resistant tumor model that is achieved via targeting tumor blood vessels by a dual-ligand installed and size-controlled liposomal system. The NGR motif peptide was employed as a specific ligand for targeting CD13, which is overexpressed in tumor blood vessels [6], and tetra-arginine (R4) was used as a CPP ligand. The advantage of targeting the neovasculature rather than RCC was verified by a direct comparison of the sensitivity to doxorubicin (DXR) in RCC and tumor endothelial cells (TECs) derived from RCC tissue. In an in vivo therapeutic study, to exclude the possibility of direct liposomal cytotoxicity to RCC, the size of liposomes was controlled, so that the liposomes were prevented from extravasation into tumor tissue via the enhanced permeability and retention (EPR) effect [12,13]. Finally, we compared the therapeutic efficacy of a dual-ligand LP and Doxil (Caelyx)®, a clinically used liposomal system for delivering doxorubicin to tumor cells via the EPR effect [14].

2. Materials and methods

2.1. Materials

Distearoyl-*sn*-glycero-3-phoshoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG-DSPE), cholesterol (Chol) and rhodamine-labeled DOPE (Rho-DOPE) were purchased from AVANTI Polar Lipids (Alabaster, AL, U.S.A.). PEG-DSPE with a functional maleimide moiety at the terminal end of PEG: *N*-[(3-maleimide-1-oxopropyl) aminopropyl polyethyleneglycol-carbamyl] distearoylphosphatidyl-ethanolamine (Mal-PEG-DSPE), egg phosphatidylcholine (EPC) and hydrogenated soybean phosphatidylcholine (HSPC) were purchased from Nippon Oil and Fat Co. (Tokyo, Japan). [^{3}H]cholesteryl hexadecyl ether (CHE) was purchased from Perkin-Elmer Life Sciences Japan. Stearylated tetraarginine (STR-R4) was purchased from PolyPeptide Laboratories (San Diego, CA, U.S.A). NGR motif peptide, CYGGRGNG was obtained from Sigma Genosys Japan (Ishikari, Japan). The NGR motif peptide was conjugated with Mal-PEG-DSPE (NGR-PEG-DSPE) as described previously [10]. Alexa 647-conjugated griffonia simplicifolia isolectin B4 (GS-IB4-Alexa647) was purchased from Invitrogen. Hoechst33342 and Cell Counting Kit-8 were purchased from DOJINDO. RPMI 1640 was purchased from Sigma (St. Louis, MO, U.S.A.). Doxorubicin (DXR) was purchased from Wako (Osaka, Japan). All other chemicals used were commercially available reagent-grade products.

2.2. Preparation of LPs

A lipid film composed of EPC/Chol/Rho-DOPE (7:3:0.1 molar ratio) was prepared by evaporation, followed by hydration with PBS. The particle size was controlled by extrusion through polycarbonate membrane filter with a pore diameter of 0.4 μm for large sized LPs, and subsequently through a 0.05 μm pore diameter for small sized LPs. To modify the prepared liposomes with STR-R4, PEG-DSPE, or NGR-PEG-DSPE, they were incubated with the indicated amounts of STR-R4, PEG-DSPE, or NGR-PEG-DSPE for 60 min at 55 °C, 950 rpm. The average diameter and the zeta-potential of the prepared liposomes were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS ZEN3600 (MALVERN Instruments, Worchestershire, U.K.).

2.3. Liposomal doxorubicin formulations

A lipid film composed of HSPC/Chol (7:3 molar ratio) was prepared by evaporation. The lipid film was hydrated with 155 mM ammonium sulfate (pH 5.5) at 65 °C, and the particle size of liposomes was controlled by extrusion. The extruded liposomes were loaded on a Sepadex-G25 gel filtration column to exchange the outer buffer to PBS (pH 7.4). DXR was incubated with the extruded liposomes (1:10 wt/wt) at 60 °C for 1 h. After removing free DXR by gel filtration, the DXR loaded liposomes were modified with STR-R4, PEG-DSPE and NGR-PEG-DSPE, as described above. The loading efficiency of DXR in liposomes was determined by measuring the fluorescence of DXR (Ex = 450 nm, Em = 590 nm) of prepared liposomes following treatment with MeOH to disrupt the liposome structure. Doxil (doxorubicin encapsulated in small sized liposomes) was prepared as described previously [15].

2.4. Cell lines and culture

Renal cell carcinoma (RCC), OS-RC-2 cells (Riken Cell Bank, Tsukuba, Japan) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml), streptomycin (100 μg/ml) at 37 °C and 5% CO_2, respectively and used within 6 months of obtaining them from Riken Cell Bank.

2.5. Animal experiments and RCC xenograft model

Four-week-old male BALB/cAJcl-*nu/nu* mice were purchased from CLEA Japan. OS-RC-2 cells (1×10^6 cells) in 70 μl PBS were s.c. inoculated into their back, and then grown until the tumor volume was 80–150 mm^3. Tumor volume was calculated using the formula: $1/2 \times a \times b^2$, where a and b represent the largest and smallest tumor diameters, respectively. All experiments were approved by the Hokkaido University Animal Care Committee.

2.6. Fluorescence confocal microscopy and determination of vessel area

Liposomes labeled with rhodamine with the indicated lipid doses were intravenously injected into tumor-bearing mice. Tumor tissues were collected after 24 h, and the endothelial cells and nucleus were then stained with GS-IB4-Alexa647 (20 μg/ml) and Hoechst 33342 (40 μM) in PBS for 1 h. Tumor tissue images were collected by a confocal laser scanning microscope (Nikon A1) equipped with a ×20 dry objective lens. The total pixels of blood vessels (green) in the tumor, liver and spleen were calculated using the ImagePro-plus software (Media Cybernetics Inc., Bethesda, MD, USA).

2.7. Biodistribution of liposomes

To evaluate the biodistribution of liposomes, the lipid membrane was labeled with [^{3}H]CHE, as a lipid phase maker. Liposomes were administered to tumor-bearing mice via the tail vein at a dose of 0.5 μmol lipid. At 24 h post-injection, the radioactivity in the blood and tissues was measured, as described previously [16]. The blood concentration and tissue accumulation of liposomes are represented as the percentage of the injected dose (ID) per milliliter of blood and %ID per gram of tissue, respectively.

2.8. In vivo therapeutic efficacy

Liposomes encapsulating DXR were intravenously administered into tumor-bearing mice with indicated doses of mg DXR/kg body weight at indicated time points. Tumor volume and body weight were monitored at 3 days intervals after the doses.

2.9. Isolation of mouse tumor endothelial cells (TECs)

TECs were isolated, as described previously [17–22]. In a typical procedure, the TECs were isolated from OS-RC-2 tumors and dermal tissue in tumor-bearing mice using a magnetic cell sorting system (MACS; Milteny Biotec, Tokyo, Japan). The TECs were plated onto 1.5% gelatin-coated culture plates and grown in EGM-2MV (Clonetics, San Diego, CA) and 15% FBS. Diphtheria toxin (500 ng/ml; Calbiochem, San Diego, CA) was added to the TEC subcultures to kill any remaining human tumor cells.

2.10. Cytotoxicity assay of RCC and TEC with free DXR

OS-RC-2 and OS-RC-2 EC were incubated in 96-well plates (5×10^3 cells/well) with free DXR at the indicated doses for 8 h. After removing DXR contained medium, cells were further cultured with fresh medium for 16 h. The cells were then incubated with fresh medium containing 10% (v/v) Cell Counting Kit-8 reagent for an additional 2 h. The absorbance (A) of each well was measured by a microplate reader (Thermo Scientific Varioskan Flash) at 450 nm. The percentage cytotoxicity is equal to [1-(A of experimental wells/A of control wells)]$\times$100.

2.11. Statistical analysis

Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the SNK test. Pairwise comparisons between treatments were made using a student's t-test. A P-value of <0.05 was considered significant.

3. Results

3.1. Effect of PEG-liposome size on the distribution in tumor

Generally, long-circulating liposomes with diameters of around 100 nm passively accumulate in tumor via the EPR effect [12,13]. This led us to assume that large sized liposomes might be suitable for vascular targeting. We first evaluated the effect of liposome size on distribution in the case of OS-RC-2 tumor tissue. PEG-LPs with an average diameter of either 100 nm or 300 nm were prepared as small PEG-LP or large PEG-LP, respectively (Fig. 1A and Table 1). After i.v. injection to tumor-bearing mice, the small PEG-LPs were mainly found to be exterior from the blood vessels (Fig. 1B). In contrast, large PEG-LPs were mainly found in close proximity to the blood vessels (Fig. 1C). These results suggest that the distribution of PEG-LPs in OS-RC-2 could be altered by controlling size of PEG-LPs, and large LPs were used in further study for developing a system that targets blood vessels.

3.2. Accumulation and distribution of a dual-ligand LP in tumor

A dual-ligand formulation was developed using large sized LPs. LPs were modified with either 10 mol% of PEG-DSPE (PEG-LP), 10 mol% NGR-PEG-DSPE (NGR-PEG-LP), or both of 10 mol% PEG-DSPE and 2.5 mol% STR-R4 (R4/PEG-LP; a dual-ligand LP), respectively. A schematic illustration of these formulations is represented in Fig. 2A and the diameters of prepared formulations were comparable, as shown in Table 1. Although the NGR motif peptide contains one arginine residue, NGR-PEG-DSPE modification had no effect on the zeta-potential of the LPs, presumably because the presence of a mono arginine residue is not sufficient to alter the zeta-potential of the liposome, which is consistent with the previously reported results [10]. Since the PEG layer masked R4, the modification with R4 had no effect on zeta-potential. A biodistribution analysis in tumor bearing-mice was performed using LPs labeled with [^{3}H]CHE. Even though neither NGR nor R4 modification showed an enhanced accumulation in tumor tissue, an increased amount of dual-ligand LP was observed in tumors compared to PEG-LP (Fig. 2B). In the case of blood and other organs, no significant difference was observed among the formulations, as shown in Supplementary Fig. S1. We further investigated the distribution of a dual-ligand LP in RCC tumor by confocal microscopy. Tumor tissues were observed at 24 h after the i.v. administration of rhodamine-labeled LPs. As shown in Fig. 2C, a few signals were detected in tumors that had been treated with PEG-LP. In the case of NGR-PEG-LP, the number of signals approached that of PEG-LP, which suggests that modification with specific NGR ligand had a minor effect on the targeting blood vessels in OS-RC-2 tumors. R4/PEG-LP showed no enhanced accumulation or distribution compared to PEG-LP, due to the fact that R4 was rendered non functional by masking by the PEG layer. However, dual modification with R4 and NGR resulted in a substantial increase in the LP signals in tumors, which suggests that the dual modification synergistically functioned to target the tumor blood vessels. These results are consistent with the biodistribution study (Fig. 2B).

3.3. Suppression of tumor growth by the dual-ligand LP

We next evaluated the anti-tumor effect of a dual-ligand LP. DXR was loaded to LPs by a pH gradient remote loading method. The encapsulation efficiency of DXR in all formulations exceeded 98%. As compared with PEG-LP, the single ligand modification showed no advantage for tumor suppression (Fig. 3). On the contrary, the dual

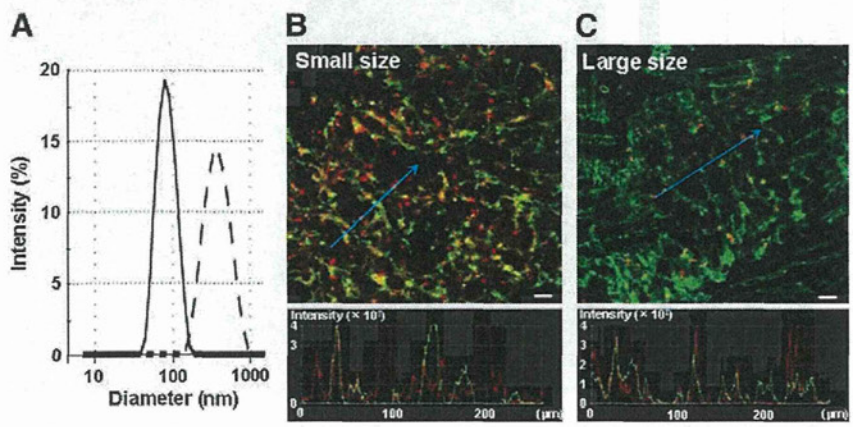


Fig. 1. Effect of liposome size on distribution in RCC tumor. (A) The size distribution of small (solid line) and large PEG-LP (dotted line) was determined by DLS measurements. (B) and (C) Upper images of unfixed tumor tissues that had been intravenously treated with either small PEG-LP or large PEG-LP (2.0 μmol lipid/mouse), respectively. Endothelial cells were labeled with lectin (green). The lower spectra were obtained from the arrow line in upper images. Bar, 80 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Physical properties of the prepared liposomes.

	Large size				Small size	
	PEG-LP	NGR-PEG-LP	R4/PEG-LP	R4/NGR-PEG-LP (dual-ligand LP)	PEG-LP	Doxil
Diameter (nm)	298 ± 29	311 ± 18	296 ± 26	304 ± 17	100 ± 17	85 ± 3
PDI	0.209 ± 0.012	0.214 ± 0.028	0.225 ± 0.008	0.215 ± 0.025	0.089 ± 0.023	0.121 ± 0.021
Zeta-potential (mV)	−13 ± 3	−16 ± 2	−11 ± 4	−11 ± 3	−13 ± 3	−11 ± 2

Data are the means ± SD of at least three different preparations.
In case of large size, molar ratio of EPC/cholesterol for biodistribution study or HSPC/cholesterol for anti-cancer study was fixed at 7:3. Large size LPs were modified with 10 mol% PEG-DSPE or NGR-PEG-DSPE, and 2.5 mol% STR-R4. In case of small size, PEG-LP for biodistribution study was composed of EPC/Chol (7:3) with 5% PEG-DSPE, and Doxil for the anti-cancer study was composed of HSPC/Chol (3:2) with 5 mol% PEG-DSPE.

ligand LP significantly depressed tumor growth. This result is in good agreement with the distribution study (Fig. 2B and C). These findings indicated that the dual-ligand formulation can be used for targeting endothelial cells in OS-RC-2 tumors after systemic administration.

3.4. Comparison of dual-ligand with Doxil (Caelyx)®

We then compared the pharmacological efficacy of a dual-ligand LP with that of Doxil (Caelyx)® which has been approved for clinical use [14]. The diameters of the Doxil particles were controlled at around 100 nm (Table 1). A biodistribution analysis showed that uptake by liver and spleen was independent and dependent on the size of liposomes respectively, consistent with previous studies [23,24]. This accounts for the lower blood concentration for the dual-ligand LP compared to Doxil. As a result of its long blood circulation, Doxil accumulated at higher levels in tumors via the EPR effect than a dual-ligand LP did via the targeting of blood vessels (Fig. 4A). Since tumor suppression by a dual-ligand LP at a dose of 1.5 mg/kg DXR was comparable to DXR dose of 6.0 mg/kg, further studies were performed using liposomal DXR at a dose of 1.5 mg/kg considering side effects and clinical use (Fig. S2). Despite an enhanced accumulation in tumors, no anti-tumor effect was observed for the systemic treatment of Doxil (Fig. 4B). By contrast, tumor growth suppression was clearly observed for the case of treatment with a dual-ligand LP. As a result of the inhibition in tumor growth, the severe body weight loss shown for PBS and Doxil was improved (Fig. 4C).

The density of blood vessels in the tumor, liver and spleen was also observed as shown in Fig. 5A. Blood vessel density was clearly diminished as the result of treatment with the dual-ligand LP, while Doxil has no effect, similar to the controls. Even though liver and spleen are major clearance organs for the prepared formulations (Fig. S1), neither damage nor an abnormal morphology of blood vessels was observed in the liver and spleen (Fig. 5A). Moreover the quantification of the area of blood vessels also showed that blood vessels were disrupted exclusively in tumor tissue (Fig. 5B). No abnormal ALT value was observed in Doxil and dual-ligand LP treated

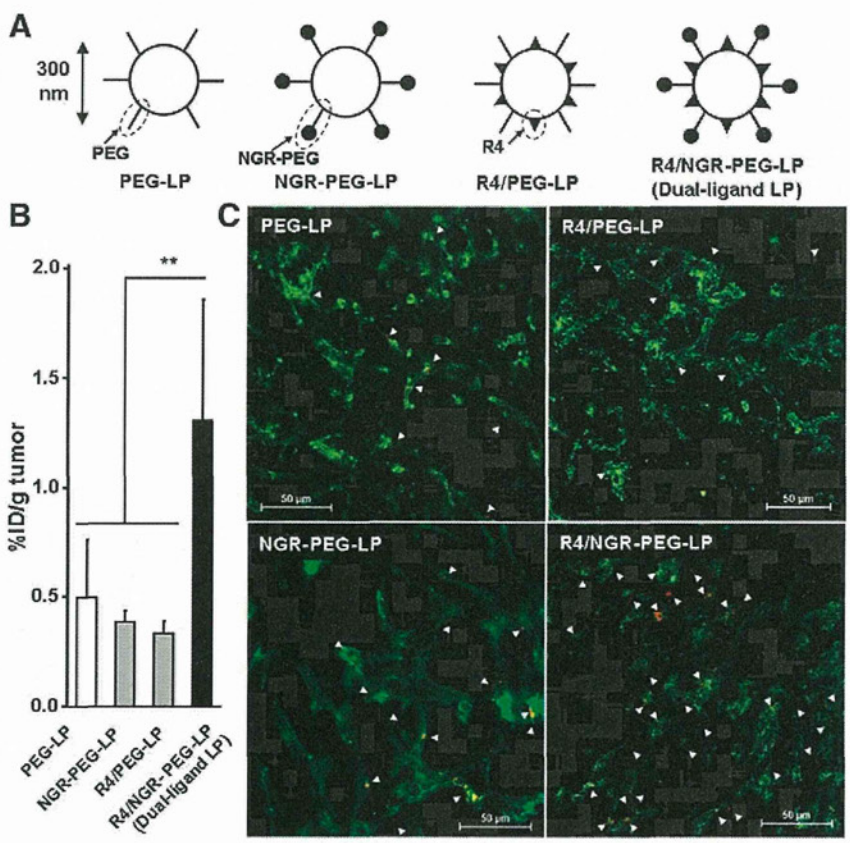


Fig. 2. Accumulation and localization of a dual-ligand LP in tumor. (A) Schematic illustration of prepared formulations. LPs of 300 nm in diameter were modified with either PEG, NGR modified PEG or R4. Dual-ligand LP was prepared by modified with both NGR modified PEG and R4. (B) Tumor accumulation at 24 h after systemic administration of formulations labeled with [^{3}H] is represented by %ID/g tissue (the mean ± SD, $n = 4$). $^{**}P < 0.01$. (C) Images of unfixed tumor tissues intravenously treated with each formulation labeled with rhodamine (0.5 μmol lipid/mouse). Tumor endothelial cells were labeled with lectin (green). Arrow heads point red signals (liposomes) along with blood vessels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

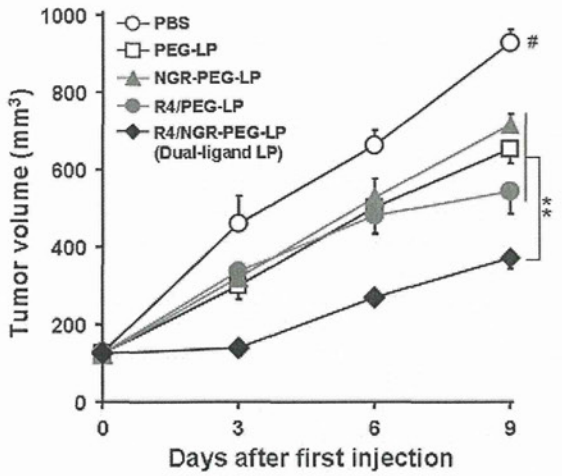


Fig. 3. Therapeutic effect of a dual-ligand LP in tumor. PBS or LPs containing 6 mg/kg of DXR were i.v.-injected on day 0 and 3. Tumor volume was monitored at indicated times. Dual-ligand LP showed better effect on the tumor growth than other formulations. $^{**}P<0.01$, $^{\#}P<0.01$ (PBS treated group versus LP-treated groups). A dual-ligand LP showed significant tumor growth suppression compared to the other formulations.

mice (Fig. S3). These results suggest that the dual-ligand LP specifically disrupted the neovasculature in OS-RC-2 tumors, but had no effect on normal endothelial cells in normal tissues such as the liver and spleen.

3.5. Comparison of cytotoxicity in tumor cell and tumor endothelial cell

To elucidate the mechanisms responsible for the improved antitumor efficacy of the dual-ligand LP compared to Doxil, we investigated the sensitivity of OS-RC-2 tumor cells and TECs to free DXR. TECs from OS-RC-2 tumor tissue were successfully collected, as described previously [17–22]. As shown in Fig. 6, the TECs were more sensitive to DXR by 2 orders of magnitude than tumor cells. The EC50 for DXR in TECs and OS-RC-2 tumor cells was calculated as 2.0 μg/ml and 95.1 μg/ml, respectively. The result strongly supports that the conclusion that the dual-ligand LP targeting TEC showed better tumor suppression than Doxil in OS-RC-2 tumor bearing mice.

4. Discussion

It is now well recognized that liposomes (LPs) constitute drug delivery vehicles that can be used in cancer therapy [25]. Long-circulating liposomes, produced by modification with poly(ethylene

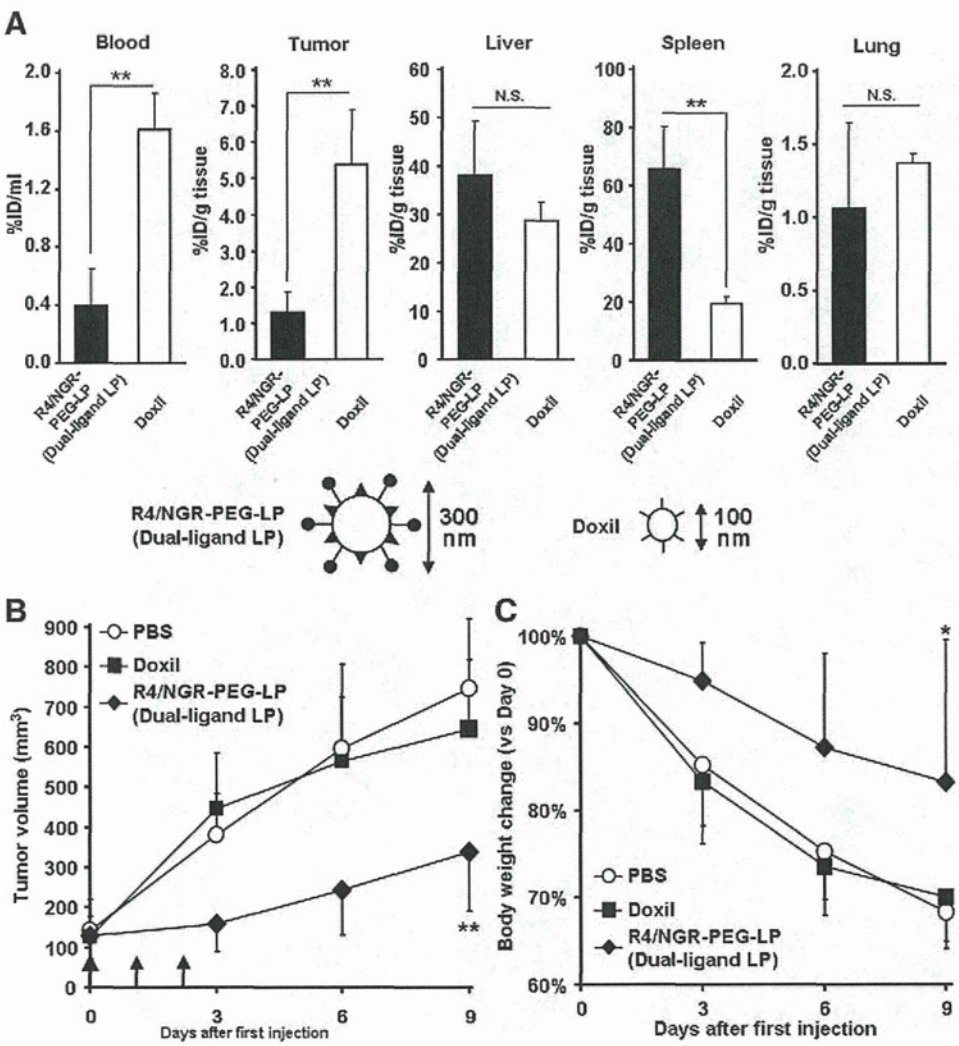


Fig. 4. Comparison of biodistribution, tumor growth and body weight change by dual-ligand LP with Doxil. (A) The biodistribution in tumor-bearing mice was determined using [^{3}H] labeled formulations. Blood concentration and tissue accumulation at 24 h after systemic administration of formulations are represented by %ID/ml and %ID/g tissue (the mean ± SD, $n=4$), respectively. $^{**}P<0.01$, N.S.: not significant difference. (B) and (C) PBS or LPs containing 1.5 mg/kg of DXR were i.v.-injected on day 0, 1 and 2. Tumor volume and body weight (the mean ± SD, $n=4$) was monitored at indicated times. Body weight change was expressed as relative change versus day 0. $^{*}P<0.05$, $^{**}P<0.01$.

glycol) (PEG) (PEG-LPs), the size of which is controlled at around 100 nm in diameter, are able to passively accumulate in tumors via the EPR effect [12,13]. Doxil (Caelyx)®, PEGylated liposomal DXR, accumulates at high level in solid tumors and has less side effects compared with free DXR, and is clinically used in the treatment of AIDS-related Kaposi's sarcoma and ovarian carcinomas [14]. To achieve further chemotherapeutic efficacy, tumor targeting PEGylated liposomes were developed by attaching ligands that specifically target molecules that are specifically expressed on tumor cells [26].

Chemotherapeutic resistance in tumor cells is a serious obstacle in cancer therapy. Renal cell carcinomas (RCCs) are one of the most resistant tumors [1]. Tumor vasculature targeting by drug vehicles is a promising strategy for overcoming the resistance of tumor cells to drugs. In the present study, we used a 300 nm diameter liposome as a platform for developing a dual-ligand system for targeting endothelial cells, of which the size is larger than the one that is usually used, as shown in Fig. 7. We initially assumed that a large size could exclude the possibility of an anti-tumor effect by liposomes that accumulated in tumors via the EPR effect. As shown in Fig. 1, only a few liposomes were observed to be located outside of blood vessels in case of large sized particles, unlike small sized liposomes.

To develop an active targeting delivery system for tumor endothelial cells (TECs), the NGR motif peptide was employed as a ligand for CD13, which is overexpressed in TECs [6]. CD13 targeting systems with the NGR motif peptide showed enhanced therapeutic efficacy in lung, ovarian carcinoma and neuroblastoma [7,8,27]. Unexpectedly, in the OS-RC-2 tumor model, modification of the NGR motif resulted in a minor effect on targetability and tumor growth (Figs. 2 and 3). One possible reason for this is that the NGR motif peptide used in the present study is a linear form of which the binding affinity is inferior to that for the cyclic form, leading to smaller amounts of liposomes being delivered. Another possible reason is that CD13 is not abundantly expressed in OS-RC-2 xenografts compared to other kinds of tumors. To induce the effect of a ligand on increasing the medicinal benefit of cargos, ligand tagged PEG-LPs should be internalized into target cells via endocytosis, followed by endosomal escape. However, specific receptor-mediated endocytosis is proceeding in a saturable manner, due to limited number of receptors and the recycling of endocytosis, which restricts the amount of liposomes that are taken up by the target cells. To overcome this limitation, we proposed the use of a dual-ligand delivery system composed of a specific ligand and a cell penetrating peptide (CPP), as a cationic ligand [10,11]. Target ligands are conjugated at the top of the PEG chain and CPPs are grafted on the surface of the liposomes so as to be masked by PEG when circulating in the blood. After the TECs recognize the specific ligand, the liposomes must resist removal from the surface of TECs under the blood flow. In the dual-ligand design, the interaction of liposomes with target TECs mediated by a target ligand is supported by a CPP

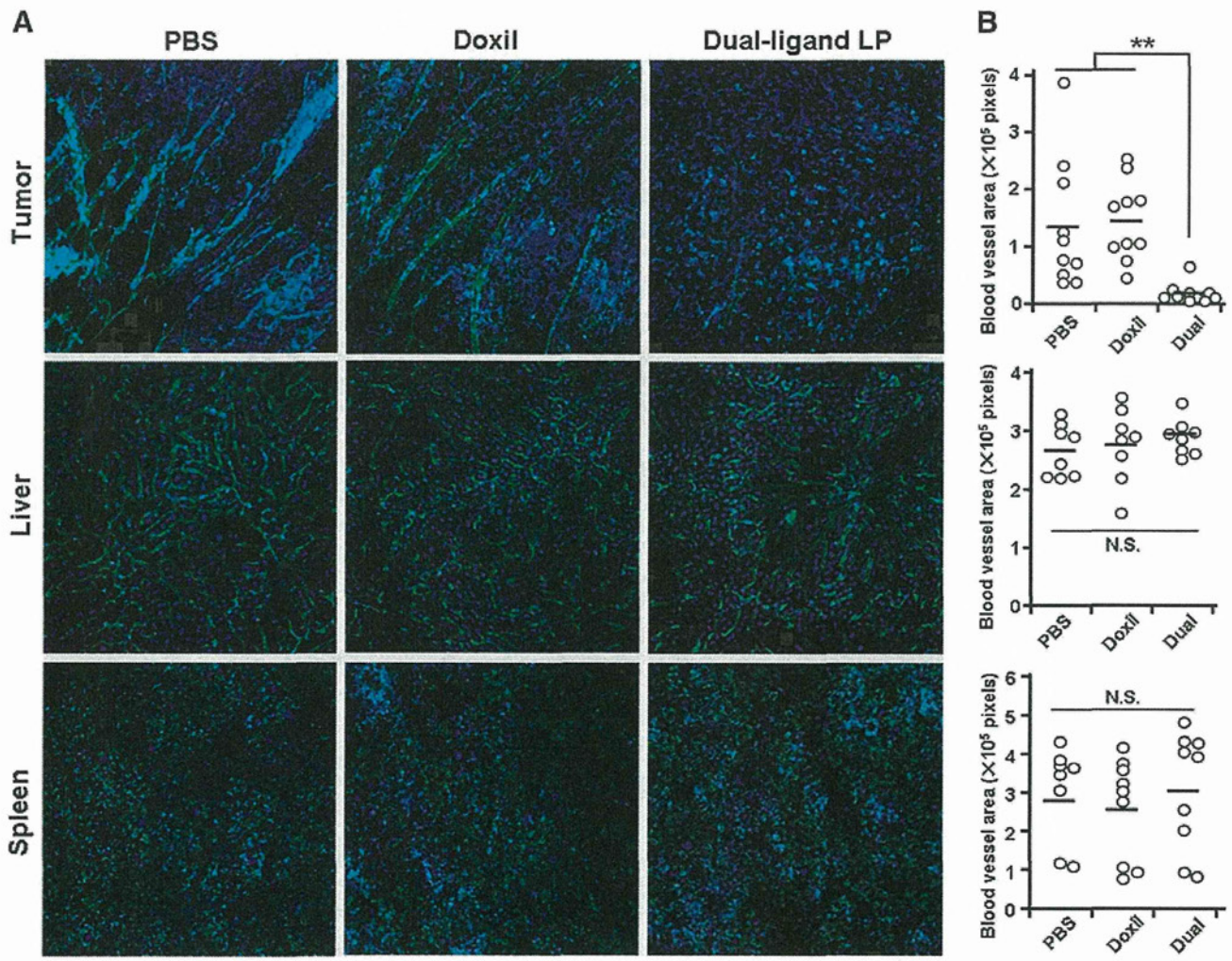


Fig. 5. Effect of cytotoxicity on blood vessels. PBS or LPs containing 1.5 mg/kg of DXR were intravenously dosed first 3 days. At 24 h after final injection, tumor, liver and spleen were collected. Endothelial cells and nucleus in unfixed tissues were stained with lectin (green) and Hoechst33342 (blue), respectively. (A) Images were captured by a confocal microscopy. (B) The pixels for endothelial cells in tumor, liver and spleen were quantified ($n = 8$–10). $^{**}P<0.01$. N.S.: not significant difference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

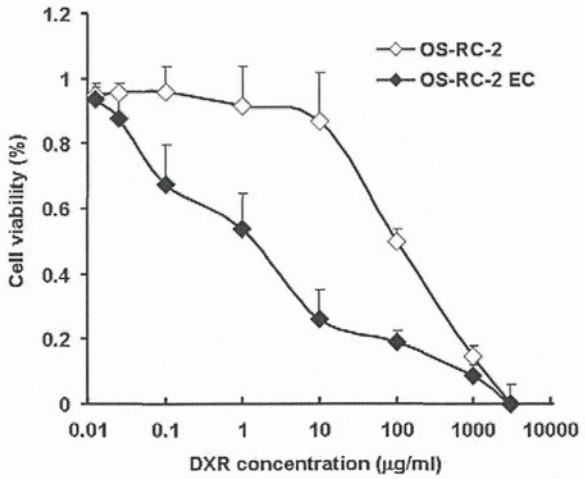


Fig. 6. Comparison of sensitivity to DXR in RCC tumor cells and TECs. OS-RC-2 cells and TECs recovered from OS-RC-2 tumor tissue were incubated with free DXR at indicated concentrations for 8 h. The cells were further incubated for 18 h, followed by cell counting. EC50 of TECs and tumor cells to DXR is 2.0 μg/ml and 95.1 μg/ml, respectively.

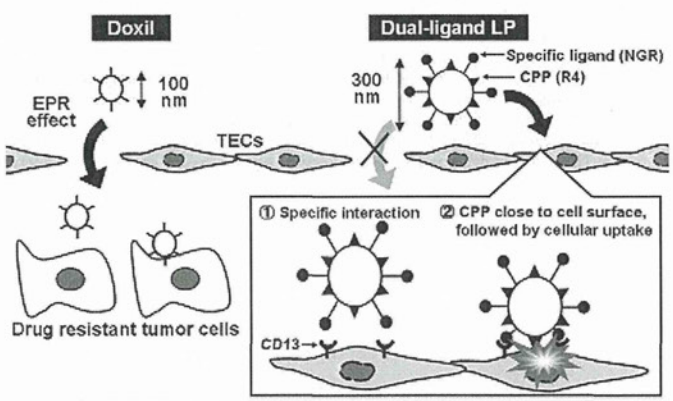
via strong cationic interactions with cell surfaces. Subsequently, the liposome is efficiently taken up by cells via the CPP, which is largely independent of the uptake mechanism associated with receptor-ligand interactions (Fig. 7) [11].

In addition, we employed a liposome with a diameter of 300 nm in the design of a dual-ligand formulation, not a 100 nm-diameter liposome, which is generally used for drug targeting to tumors [7–9]. We hypothesized that a large size would be more advantageous for targeting tumor endothelial cells than a small size by preventing the liposomes from extravasation to the tumor through permeable tumor blood vessels and would allow the liposomes to efficiently recognize the blood vessels. As we expected, PEGylated liposomes with diameters of 300 nm were detected mainly along the blood vessels, while PEGylated liposomes with a diameter of 100 nm were distributed both inside and outside of the blood vessels (Fig. 1). We also compared the distribution of large and small dual-ligand LPs. As shown in Fig. S4, the distribution for dual-ligand LPs in tumor tissues was well correlated with that for PEGylated liposomes (Fig. 1).

Additionally, it is possible that large liposomes represent an advantage for ligands to target vascular walls rather than small ones. It was reported that particles with diameters >200 nm appear to be more effective in adhering firmly to the margins of vascular walls under flow than particles with diameters of <200 nm [28]. Therefore, the cellular binding affinity of a large dual-ligand LP was evaluated in comparison with the small one. As shown in Fig. S5, the relative K_d value of a large dual-ligand LP was around 10 times lower than that of the small one. This might account for this enhancement, since a large dual-ligand LP with a large surface would allow its ligands to interact more frequently with target molecules than a smaller sized particle, which would result in multivalent and efficient binding. These results suggest that a large sized liposome would seem to be preferred for the dual-ligand formulation than a small sized one. To further clarify the advantage of a large size for dual-ligand mediated targeting, we also compared the tumor suppression of a dual-ligand LP with a diameter of 300 nm and with a 100 nm diameter dual-ligand LP. As shown in Fig. S6, tumor growth suppression by the large dual-ligand LP was slightly superior compared to the small dual-ligand LP, even though higher amounts of a small dual-ligand LP were found in the tumor. Whereas the large size represented disadvantage regarding the increased accumulation of liposomes in the spleen (Fig. 4A), no serious side effects were observed (Figs. 5 and S3). Taking these results into consideration, a large liposome appears to be preferred for a dual-ligand formulation to target tumor endothelial cells than a small sized particle; therefore further evaluations were performed using a large-diameter dual-ligand LP.

A dual-ligand LP of 300 nm encapsulating DXR represented an enhanced anti-tumor effect compared to Doxil (Fig. 4B). If 1 g tumor tissue contains 10^8 cells [29], we estimate that the availability of DXR in tumor cells would be 5% ID/10^8 cells, since the amount of liposome in a tumor via the EPR effect was approximately 5% ID/g tumor (Fig. 4A). On the other hand, tumor endothelial cells constitute only approximately 2% of the total tumor tissue (2×10^6 cells/g tumor) [18]. Because 1.5% ID/g tumor of liposomes was found in the case of the dual-ligand LP, the availability of DXR in TEC is calculated as 0.75% ID/10^6 cells, which means that the concentration of DXR in TEC would be expected to be at least 10-fold higher than that in OS-RC-2 cells. Furthermore, cytotoxicity analyses indicated that TECs derived from OS-RC-2 tissue are approximately 2 orders more sensitive to DXR than OS-RC-2 cells (Fig. 6). Taking these facts into consideration, targeting TECs should be around 3 orders of magnitude more efficient in terms of exerting cytotoxicity by DXR than targeting OS-RC-2 kidney cancer tissue (Fig. 7). Even though a dual-ligand LP efficiently disrupted blood vessels, tumor growth was partially inhibited, presumably because surviving OS-RC-2 cells could generate new blood vessels. For further therapeutic efficacy, the inhibition of factors such as VEGF from OS-RC-2 cells should be used in combination with the above described therapy.

In summary, here we report on a novel anti-neovasculature therapy for drug-resistant renal cell carcinomas based on a unique delivery system comprised of large-sized liposomes that had been modified with a dual-ligand. We also directly compared the cytotoxicity between tumor cells and tumor endothelial cells. The findings clearly show that targeting the neovasculature is 3-orders more efficient than tumor cells in a drug resistance tumor. The results provide a promising basis for further anti-angiogenic chemotherapy, which may be valuable for future clinical applications for drug-resistant cancer.



Tumor cells	Target cells	TECs
1×10⁸ cells/g tumor	No. of target cells	2×10⁶ cells/g tumor
95.1 μg/mL	Sensitivity to DXR (EC50)	2.0 μg/mL
2380	Relative required dose	1
5% ID/g tumor	Delivered amount	1.5 %ID/g tumor

Fig. 7. Schematic diagram of the strategy used to develop the dual-ligand LP. Doxil accumulates in tumors via the EPR effect. The size of a dual-ligand LP is controlled around 300 nm and specific ligands and CPPs are modified on the top of PEG chain and on the surface of liposomes, respectively. CPPs should not be functional and free from oposonins due to steric hindrance of the PEG layer in the blood circulation. While after arriving at the target tumor endothelial cells, cellular association via the specific ligands (1) allows CPPs to exert their powerful ability to internalize the liposomes into cells due to proximity of the liposomes to the surface of target cells (2). 1 g of tumor tissue contains 10^8 cells, and tumor endothelial cells constitute approximately 2% of tumor tissue. Therefore, the relative required dose of DXR by targeting OS-RC-2 is estimated approximately 2380 fold higher than that by targeting tumor endothelial cells to kill the objective cells. Despite the differences, the delivery of Doxil is only 3-fold larger than the dual-ligand LP, which clearly accounts for the absence of an anti-tumor effect of Doxil in RCC tumor.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.06.019.

References

[1] N.J. Vogelzang, W.N. Stadler, Kidney cancer, Lancet 352 (1998) 1691–1696.
[2] E. Soto-Vega, C. Arroyo, Y. Richaud-Patin, M. García-Carrasco, L.G. Vázquez-Lavista, L. Llorente, P-glycoprotein activity in renal clear cell carcinoma, Urol. Oncol. 27 (2009) 363–366.
[3] T.E. Hutson, Targeted therapies for the treatment of metastatic renal cell carcinoma: clinical evidence, Oncologist 16 (2011) 14–22.
[4] G.C. Lorenzo, C. Buonerba, M. Biglietto, F. Scognamiglio, B. Chiurazzi, F. Riccardi, G. Carteni, The therapy of kidney cancer with biomolecular drugs, Cancer Treat. Rev. 36 (2010) S16–S20.
[5] J. Stevens, B. St. Croix, Tumor endothelial markers, In: in: W.D. Figg, J. Folkman (Eds.), Angiogenesis: An Integrative Approach from Science to Medicine, Springer, 2008, pp. 333–342.
[6] W. Arap, R. Pasqualini, E. Ruoslahti, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model, Science 279 (1998) 377–380.
[7] F. Pastorino, C. Brignole, D. Di Paolo, B. Nico, A. Pezzolo, D. Marimpietri, G. Pagnan, F. Piccardi, M. Cilli, R. Longhi, D. Ribatti, A. Corti, T.M. Allen, M. Ponzoni, Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy, Cancer Res. 66 (2006) 10073–10082.
[8] E.A. Murphy, B.K. Majeti, L.A. Barnes, M. Makale, S.M. Weis, K. Lutu-Fuga, W. Wrasidlo, D.A. Cheresh, Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 9343–9348.
[9] Y. Murase, T. Asai, Y. Katanasaka, T. Sugiyama, K. Shimizu, N. Maeda, N. Oku, A novel DDS strategy, "dual-targeting," and its application for antineovascular therapy, Cancer Lett. 287 (2010) 165–171.
[10] K. Takara, H. Hatakeyama, N. Ohga, K. Hida, H. Harashima, Design of a dual-ligand system using a specific ligand and cell penetrating peptide, resulting in a synergistic effect on selectivity and cellular uptake, Int. J. Pharm. 396 (2010) 143–148.
[11] G. Kibria, H. Hatakeyama, N. Ohga, K. Hida, H. Harashima, Dual-ligand modification of PEGylated liposomes shows better cell selectivity and efficient gene delivery, J. Control. Release 153 (2011) 141–148.
[12] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[13] H. Hatakeyama, H. Akita, H. Harashima, A multifunctional envelope type nano device (MEND) for drug and gene delivery to tumors based on the EPR effect: a strategy for overcoming the PEG dilemma, Adv. Drug Deliv. Rev. 63 (2011) 152–160.
[14] M.E. Davis, Z. Chen, D.M. Shin, Nanoparticle therapeutics: an emerging treatment modality for cancer, Nat. Rev. Drug Discov. 7 (2008) 771–782.
[15] T.A. ElBayoumi, V.P. Torchilin, Tumor-specific anti-nucleosome antibody improves therapeutics efficacy of doxorubicin-loaded long-circulating liposomes against primary and metastatic tumor in mice, Mol. Pharm. 6 (2009) 246–254.
[16] H. Hatakeyama, H. Akita, K. Maruyama, T. Suhara, H. Harashima, Factor governing the in vivo tissue uptake of transferring coupled polyethylene glycol liposomes in vivo, Int. J. Pharm. 281 (2004) 25–33.
[17] K. Hida, Y. Hida, D.N. Amin, A.F. Flint, D. Panigrahy, C.C. Morton, M. Klagsbrun, Tumor-associated endothelial cells with cytogenetic abnormalities, Cancer Res. 15 (2004) 8249–8255.
[18] N. Ohga, K. Hida, Y. Hida, C. Muraki, K. Tsuchiya, K. Matsuda, Y. Ohiro, Y. Totsuka, M. Shindoh, Inhibitory effects of epigallocatechin-3 gallate, a polyphenol in green tea, on tumor-associated endothelial cells and endothelial progenitor cells, Cancer Sci. 100 (2009) 1963–1970.
[19] K. Matsuda, N. Ohga, Y. Hida, C. Muraki, K. Tsuchiya, T. Kurosu, T. Akino, S.C. Shih, Y. Totsuka, M. Klagsbrun, M. Shindoh, K. Hida, Isolated tumor endothelial cells maintain specific character during long-term culture, Biochem. Biophys. Res. Commun. 394 (2010) 947–954.
[20] T. Akino, K. Hida, Y. Hida, K. Tsuchiya, D. Freedman, C. Muraki, N. Ohga, K. Matsuda, K. Akiyama, K. Harabayashi, M. Klagsburn, M. Shindoh, Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors, Am. J. Pathol. 175 (2009) 2657–2667.
[21] C. Muraki, N. Ohga, Y. Hida, H. Nishihara, Y. Kato, K. Tsuchiya, K. Matsuda, Y. Totsuka, M. Shindoh, K. Hida, Cyclooxygenase-2 inhibition causes antiangiogenic effects on tumor endothelial and vascular progenitor cells, Int. J. Cancer 130 (2012) 59–70.
[22] T. Kurosu, N. Ohga, Y. Hida, N. Maishi, K. Akiyama, W. Kakuguchi, T. Kuroshima, M. Kondo, T. Akino, Y. Totsuka, M. Shindoh, F. Higashino, K. Hida, HuR keeps an angiogenic switch on by stabilising mRNA of VEGF and COX-2 in tumour endothelium, Br. J. Cancer 104 (2011) 819–829.
[23] K. Maruyama, T. Yuda, A. Okamoto, S. Kojima, A. Suginaka, M. Iwatsuru, Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol), Biochim. Biophys. Acta 1128 (1992) 44–49.
[24] D. Liu, A. Mori, L. Huang, Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM_1-containing liposomes, Biochim. Biophys. Acta 1104 (1992) 95–101.
[25] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. 4 (2005) 145–160.
[26] T.M. Allen, Ligand-targeted therapeutics in anticancer therapy, Nat. Rev. Cancer 2 (2002) 750–763.
[27] Y. van Hensvergen, H.J. Broxterman, Y.W. Elderkamp, J. Lankelma, J.C. Beers, M. Heijn, B. Epie, K. Hoekman, H.M. Pinedo, A doxorubicin-CNGRC-peptide conjugate with prodrug properties, Biochem. Pharmacol. 63 (2002) 897–908.
[28] F. Gentile, A. Curcio, C. Indolfi, M. Ferrari, P. Decuzzi, The margination propensity of spherical particles for vascular targeting in the microcirculation, J. Nanobiotechnol. 6 (2008) 1–9.
[29] J.Y. Zheng, D. Chen, J. Chan, D. Ui, E. Ko, S. Pang, Regression of prostate cancer xenografts by a lentiviral vector specifically expressing diphtheria toxin A, Cancer Gene Ther. 10 (2003) 764–770.