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In vitro and *in vivo* evaluation of a paclitaxel conjugate with the divalent peptide E-[c(RGDfK)$_2$] that targets integrin $\alpha_v\beta_3$

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ABSTRACT

The $\alpha_v\beta_3$ integrin is overexpressed on proliferating endothelial cells such as those present in growing tumors as well as on tumor cells of various origins. Tumor-induced angiogenesis can be inhibited *in vivo* by antagonizing the $\alpha_v\beta_3$ integrin with small peptides containing the arginyl-glycyl-aspartic acid (RGD) amino acid sequence. The divalent cyclic peptide E-[c(RGDfK)$_2$] is a novel ligand-based vascular-targeting agent that binds integrin $\alpha_v\beta_3$ and demonstrated high uptake in OVCAR-3 xenograft tumors. In this work, we coupled the 2′-OH-group of paclitaxel through an aliphatic ester to the amino group of E-[c(RGDfK)$_2$] or the control peptide c(RADfK), thus obtaining the derivatives E-[c(RGDfK)$_2$]-paclitaxel and c(RADfK)-paclitaxel. Subsequently, we investigated the activity of the paclitaxel derivatives using several well-established *in vitro* angiogenesis assays: using a standard 72 h endothelial cell proliferation assay, we showed that both E-[c(RGDfK)$_2$]-paclitaxel and c(RADfK)-paclitaxel inhibit the proliferation of human umbilical vein endothelial cells (HUVEC) in a similar manner as free paclitaxel (IC_{50} value ~0.4 nM), an observation that can be explained by the half-life of the paclitaxel ester bond in the conjugates of ~2 h at pH 7. In contrast, a 30-min exposure of the cells to the three drugs showed a clear difference between free paclitaxel, E-[c(RGDfK)$_2$]-paclitaxel and c(RADfK)-paclitaxel with IC_{50} values of 10 nM, 25 nM, and 60 nM, respectively. These differences are very likely due to the different routes of cellular entry of these three molecules. While the hydrophobic paclitaxel diffuses rapidly through the cell membrane, the charged peptide-containing derivative E-[c(RGDfK)$_2$]-paclitaxel binds to the overexpressed $\alpha_v\beta_3$ integrin in order to enter the cells via receptor-mediated endocytosis. The differences between the derivatives were further demonstrated using an endothelial cell adhesion assay. Inhibition of cell attachment was observed only with the E-[c(RGDfK)$_2$]-paclitaxel derivative indicating its specificity to the growing endothelial cells. Furthermore, E-[c(RGDfK)$_2$]-paclitaxel inhibited both endothelial cells migration and capillary-like tube formation. These results further demonstrate their antiangiogenic properties. *In vivo* studies in an OVCAR-3 xenograft model demonstrated no antitumor efficacy for either E-[c(RGDfK)$_2$] or E-[c(RGDfK)$_2$]-paclitaxel compared to moderate efficacy for paclitaxel.

1. Introduction

Integrins have been intensely studied as endothelial targets for antiangiogenic therapy and for vascular targeting strategies (Temming et al., 2005; Tucker, 2006). Integrins are heterodimeric cell surface receptors of which several such as integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ are overexpressed in the blood vessels of solid tumors and mediate adhesion between cells and the extracellular matrix thus promoting tumor cell migration and tumor growth. A characteristic feature of integrins is their high binding affinity for arginyl-glycyl-aspartic acid (RGD) sequences exposed on endogenous or exogenous ligands. Kessler and colleagues have developed a series of cyclic RGD peptides that interact with integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Aumailley et al., 1991; Dechantsreiter et al., 1999; Haubner et al., 1996; Pfaff et al., 1994). One of these, cyclo(RGDfV) (Cilengitide), was chosen as a clinical candidate after demonstrating antiangiogenic and antitumor efficacy in preclinical tumor models (Buerkle

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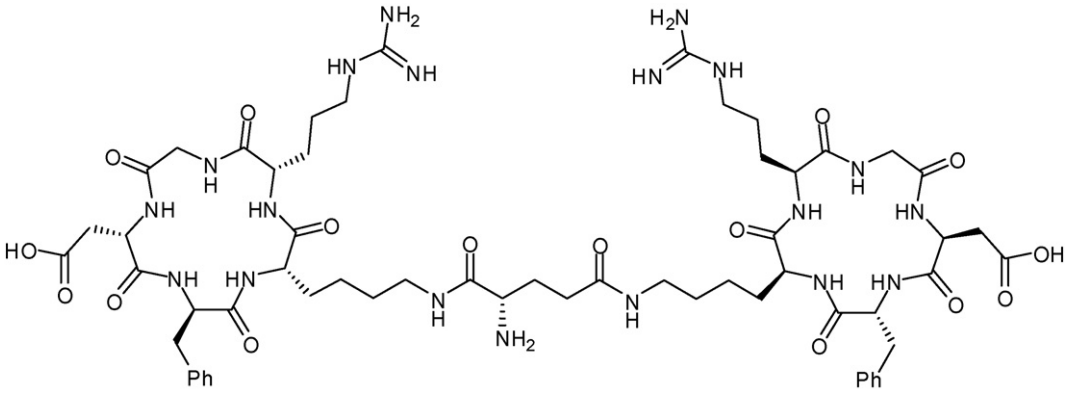

Fig. 1. Structure of E-[c(RGDfK)$_2$].

et al., 2002; MacDonald et al., 2001). In clinical trials, Cilengitide has shown antitumor efficacy against glioblastoma (Friess et al., 2006; Nabors et al., 2007), but no improvement in treating pancreas carcinoma in combination with gemcitabin compared to gemcitabin alone (Friess et al., 2006). A number of phase II trials are currently ongoing.

A potential disadvantage of integrin $\alpha_v\beta_3$ antagonists is their insufficient anti-proliferative effect on endothelial cells and their poor bioavailability necessitating fairly large doses for human use due to a short half-life of only a few hours. Vascular targeting agents that consist of a targeting moiety and an effector molecule are a possibility of improving the therapeutic efficacy of integrin $\alpha_v\beta_3$ binding ligands. For this purpose a stable peptide derivative c(RGDfK) based on the structure of Cilengitide has been developed that shows specific tumor targeting properties (Janssen et al., 2002a,b, 2004). The presence of the glutamic acid residue makes it an ideal ligand for further chemical conjugation with diagnostic or therapeutic agents. In further diagnostic studies, Janssen et al. demonstrated that a dimeric form of c(RGDfK) (Fig. 1), i.e. E-[c(RGDfK)$_2$], had improved tumor targeting properties over the monomeric form (Janssen et al., 2002b; Burkhart et al., 2004). Subsequent biodistribution studies with radiolabeled E-[c(RGDfK)$_2$] showed an uptake of up to 7.5% injected dose/g in OVCAR-3 xenograft tumors (Janssen et al., 2002b).

A tyrosine derivative of E-[c(RGDfK)$_2$], i.e. E-[c(RGDyK)$_2$], that can be conveniently radiolabeled with ^{125}I, was used for developing a E-[c(RGDyK)$_2$]-paclitaxel derivative in which paclitaxel was bound at the 2′-OH-position through a succinate spacer to the α-amino position of the glutamic acid residue of E-[c(RGDyK)$_2$] (Chen et al., 2005). This conjugate showed integrin $\alpha_v\beta_3$ binding, a threefold higher IC_{50} value in a 48-h assay against MDA-MB-435 breast cancer cells and a maximum tumor uptake of the radiolabeled E-[c(RGDyK)$_2$]-paclitaxel derivative 2 h post-injection (∼2.7% ID/g) in the MDA-MB-435 xenograft model, a value comparable to radiolabeled E-[c(RGDyK)$_2$] (∼2.4% ID/g) run as control in this experiment. No stability was reported for the ester bond, but premature release of paclitaxel was mentioned by Temming et al. when using the succinate ester of paclitaxel for their development of RGD-paclitaxel albumin conjugates (Temming et al., 2005). *In vivo* data of the E-[c(RGDyK)$_2$]-paclitaxel conjugate showing an antitumor effect were recently published (Cao et al., 2008).

As part of our ongoing projects on passive and active targeting with prodrugs (Kratz et al., 2008), we wanted to examine, in more detail, the *in vitro* properties of the paclitaxel succinate ester derivative with E-[c(RGDfK)$_2$] and the control peptide c(RADfK) that does not bind to integrin $\alpha_v\beta_3$. In this work, we report on their cytotoxicity in short- and long-term incubation experiments with human umbilical vein endothelial cells (HUVEC) as well as on their activity in a cell adhesion, migration, and capillary-like tube formation assay. Additional *in vivo* results in an OVCAR-3 xenograft model are presented.

2. Materials and methods

2.1. Materials, methods, and spectroscopy

Paclitaxel was purchased from Yick-Vic (Hong Kong, PRC); E-[c(RGDfK)$_2$] and c(RADfK) were purchased from Peptides International (Louisville, KY, USA); organic solvents: HPLC grade (Labscan Ltd., Dublin, Ireland; Roth, Karlsruhe, FRG; Merck, Darmstadt, FRG). The aliphatic paclitaxel-NHS-ester was prepared by a modified procedure according to the literature (Deutsch et al., 1989; Luo and Prestwich, 1999). All other chemicals used were at least reagent grade and obtained from Sigma–Aldrich (Deisenhofen, FRG) or Merck and used without further purification; buffers were vacuum-filtered through a 0.2-μm membrane (Sartorius, FRG) and thoroughly degassed with nitrogen prior to use. ^{1}H, ^{13}C NMR: Bruker AM 400 (internal standard: TMS); analytical HPLC and the stability study were performed with a Kontron 422 pump and a Kontron 430 detector (at 220 nm). For peak integration Geminyx software (version 1.91 by Goebel Instrumentelle Analytik, FRG) was used; column: Machery-Nagel, 100 Å, Nucleosil 100-5 C18 [4 mm × 250 mm] with pre-column WAT106166; chromatographic conditions for the analytical HPLC: flow: 1.0 mL/min, mobile phase A: AcN/0.05% aqu. TFA (30/70, v/v), mobile phase B: AcN/0.05% aqu. TFA (70/30, v/v), gradient: 0–1.5 min 100% mobile phase A; 1.5–40 min increase to mobile phase B; 40–46.5 min decrease to initial mobile phase A; injection volume: 20 μL. Chromatographic conditions for the stability study: flow: 1.0 mL/min, mobile phase A: AcN/0.05% aqu. TFA (5/95, v/v), mobile phase B: AcN/0.05% aqu. TFA (70/30, v/v), gradient and injection volume: as described above. Lyophilization was performed overnight at −40 °C with a Christ Alpha 2–4 (Gefriertrocknungs GmbH, FRG) lyophilizer. MALDI-TOF mass spectra were acquired on a Reflex III mass spectrometer (Bruker Daltonik GmbH, Bremen, FRG) in the reflector mode (positively charged ions) with external calibration. Samples were prepared with an adapted thin-layer technique (Kussmann et al., 1997). α-Cyano-4-hydroxycinnamic acid (97%, Aldrich, Taufkirchen, FRG) was used as matrix and nitrocellulose (Biorad, München, FRG, Trans-Blot Transfer Medium) as additive. The samples were washed with 0.1% TFA. Monoisotopic peaks were used for data analysis. ESI-TOF mass spectra were acquired on an Agilent 6210 system, consisting of an Agilent 1100 HPLC system with a diode array detector and an ESI-MSD TOF by Agilent Technologies (Böblingen, FRG).