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Re-programming pullulan for targeting and controlled release of doxorubicin to the hepatocellular carcinoma cells

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ABSTRACT

A novel bioconjugate for hepatocellular carcinoma (HCC) targeting was obtained by pullulan re-programming, which involves the backbone oxidation and conjugation of targeting peptide and doxorubicin (Doxo) through a releasable linker. Preliminary in vivo studies showed that the oxidation of 40 glucopyranose units (GPU) out of 100 remarkably reduced the pullulan unspecific liver tropism. This oxidized polymer was functionalized with PreS1 to selectively target the HCC and with rhodamine (Rhod) as label to perform in vitro cell up-take investigations. PreS1 and Rhod were conjugated to the aldehydes present along the oxidized pullulan backbone through a 3.4 and 2 kDa PEG spacer, respectively, and by reductive amination. The resulting PreS1-Pull-Rhod contained a mean of 8 PreS1 per oxidized pullulan chain. Cell culture studies were performed by using HepG2/SERPINB3 cells that overexpress the serpine B3 receptor and control HepG2/EMPTY cells that do not overexpress the receptor. A comparative study by cytofluorimetry and confocal microscopy performed using PreS1-Pull-Rhod and Pull-Rhod (control polymer) showed that PreS1 conveys to the conjugate high cell selectivity. Afterwards, the oxidized pullulan was exploited to generate a targeted drug delivery system by conjugation of Doxo to the polymer backbone through a hydrazone pH-sensitive bond and NH_2-$PEG_{3.4\ kDa}$-PreS1. The PreS1-Pull-Doxo conjugate showed a two-fold increase of anticancer activity with respect to the control Pull-Doxo towards HepG2/SERPINB3 cells.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancer worldwide and the second and sixth causes of cancer-related death for men and women, respectively (Zhang et al., 2016; Forner et al., 2012; Jemal et al., 1999). HCC onset is very often correlated to a variety of previously established chronic liver diseases. Notably, liver cirrhosis as well as hepatitis B and C viral infections are the predominant risk factors of this type of cancer (El-Serag, 2012). The long latency period before its diagnosis is negatively affecting the efficacy of the therapeutic treatments of HCC; during latency, in fact, most patients develop the intermediate or advanced stage of tumor progression (Fitzmorris et al., 2015). When HCC is diagnosed in the early-stages, liver transplantation, surgical resection or percutaneous ablation are first choice treatments. On the contrary, only palliative treatments are recommended by the clinical practice for tumors in intermediate and advanced stages. Chemotherapeutic treatment with sorafenib is, in most cases, non-curative and it suffers from a number of severe side effects deriving from the lack of selectivity towards cancer cells (Mauer et al., 2015; Crissien and Frenette, 2014).

Thus, efficient and reliable therapies for HCC treatment that enhance the quality of life and overall survival of patients are yet to come. Many efforts have been dedicated to design and develop new drug delivery systems with the aim to improve the selective delivery of anticancer drugs to hepatocellular carcinoma cells while sparing the exposure of the other cellular components of the liver to chemotherapeutic agents. These systems must possess suitable features that combine passive localization within the tumor interstitium according to the Enhanced Permeability and Retention (EPR) effect (Matsumura and Maeda, 1986), which limits the exposure of healthy tissues and organs to the drug, and biorecognition of the hepatocellular cancer cells by specific ligands. Notably, while drug biodistribution to the liver is not per se a difficult task to be pursued due to the physiological clearance activity of this organ, the specific targeting of hepatocellular carcinoma remains a challenge.

In this contest, polymer therapeutics are promising tools for the treatment of HCC since their physico-chemical features can be

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tuned in order to ensure improved pharmacokinetics profile with prolonged circulation times, which reduce the unspecific accumulation in the liver (Polyak et al., 2013; Duncan, 2014). In particular, polysaccharides have proved to be promising drug carriers because they present high water solubility, biocompatibility and biodegradability as well as multiple anchoring points for drugs and targeting agents (Liu et al., 2008). Among the polysaccharides, pullulan, a natural polysaccharide comprising three α-1, 4-linked glucose molecules that are repeatedly polymerized at α-1, 6-linkages on terminal glucoses, has been shown to be a versatile polymer for a variety of biomedical applications, including drug delivery and tissue engineering (Leathers, 2003). Pullulan has been so far explored for the generation of colloidal systems such as self-assembling hydrophobized nanoparticles (Lee et al., 2012) micelles (Sarika et al., 2015; Wang et al., 2014) and targeted polymer therapeutics (Scomparin et al., 2011; Bonzi et al., 2015) for the delivery of anticancer drugs.

Despite these intriguing potentials for therapeutic applications, pullulan possesses a few limitations. Pullulan was reported to accumulate in the liver at significantly higher ratio than other water-soluble polymers (Yamaoka et al., 1995; Yamaoka et al., 1993). The endocytosis of pullulan by isolated liver parenchymal cells was inhibited by known ligands of asialoglycoprotein receptors (ASGPR) suggesting ASGPR-mediated uptake (Kaneo et al., 2001). Notably, ASGPR are predominantly present on the sinusoidal cell membrane of hepatocytes and internalize sugars such as galactose or lactose and glycoproteins with terminal galactose or N-acetylgalactosamine by endocytosis (Wu et al., 2002), which may be responsible for the hepatotropic behaviour of pullulan and the endocytosis of pullulan based carriers by healthy hepatocytes. Thus ASGPR binding may obstacle any cancer cell targeting strategy using pullulan. In order to exploit this polysaccharide as delivery vehicle to selectively target the hepatocellular carcinoma cells, we have first "de-programmed" its natural ability to bind ASGPR, which is the major cause for its liver tropism. Preliminary in vivo studies have clearly shown that the hepatic accumulation of pullulan was significantly reduced upon selective oxidative treatment of this polysaccharide (Bruneel and Schacht, 1993). This chemical modification is a suitable approach to modulate the physico-chemical properties of polysaccharides, namely flexibility or conformational structure, and also their biopharmaceutical features (Kristiansen et al., 2010).

After "de-programming" the hepatotropic feature of pullulan, we instructed the activated material for specific hepatocellular carcinoma cell biorecognition. A wide range of targeting moieties is currently being investigated to target selectively different tumors. Peptides having sequences with <50 amino acids are rising a great interest due to the advantage to be smaller in size, more stable and easily produced and conjugated with respect to antibody and antibody-fragments (Bertrand et al., 2014). In 1986, Neurath et al. (1986) reported that the synthetic peptide preS1$^{21\text{-}47}$, which includes the 21-47 amino acid sequence of the original preS1 envelope protein of hepatitis B virus (HBV), is able to bind to HepG2 cells and inhibit virus entry. Many studies have described the crucial role of preS1 peptide sequence in HBV infectivity (Glebe and Urban, 2007; Meier et al., 2013). Recently, the squamous cell carcinoma antigen (SCCA), named also SERPINB3, that mediates apoptosis resistance, cellular proliferation, epithelial-to-mesenchymal transition, increase of cellular invasiveness (Turato et al., 2012; Montagnana et al., 2015) and HBV virus cell internalization (Hao et al., 2012), was found to be overexpressed by HCC cells and, importantly, to interact with peptide preS1$^{21\text{-}47}$ (Moore et al., 2003; Ruvoletto et al., 2004). Studies have shown that SERPINB3 receptor is localized both in the cytosol, not associated intimately with membrane-bound organelles or cytoskeletal structures, and on the cell surface suggesting a role as a functional surface receptor responsible for HBV binding to and internalization into human liver cells (Ruvoletto et al., 2004).Thus, preS1 has been suggested to be a suitable ligand to selectively target HCC cells through their overexpressed SERPINB3.

We aimed here to design a novel polysaccharide based bioconjugate for the selective delivery of doxorubicin to hepatocellular carcinoma. Pullulan has been selected as polymeric backbone to be suitably "re-programmed" for HCC biorecognition by the targeting agent preS1$^{21\text{-}47}$. The targeted polymer was first labelled with rhodamine to investigate the selective uptake by HCC cells overexpressing SERPINB3 receptor. Rhodamine was then replaced by doxorubicin that was conjugated through a pH-releasable bond to explore the therapeutic activity of targeted drug loaded pullulan conjugates.

2. Materials and methods

Pullulan (110 kDa, 2.25 PDI) was purchased from Sigma-Aldrich (St. Louis, USA). All the other chemical reagents including salts and solvents of analytical grade were obtained from Sigma-Aldrich (St. Louis, USA), VWR (Milan, Italy) or Carlo Erba (Milan, Italy). Diaminopolyethylene glycol 2 kDa [$PEG_{2\ kDa}(NH_2)_2$] was purchased from Iris Biotech GmbH (Marktredwitz, Germany). 3.4 kDa tBoc-amine-PEG hydroxysuccinimidyl ester [tBoc-NH-$PEG_{3.4\ kDa}$-NHS] and 3 kDa methoxy-PEG-amino ($mPEG_{3\ kDa}$-NH_2) were furnished by JenKem Technology USA (Plano, USA). Amido-protected C-terminal peptide PreS1 derivative terminating with an additional glycine at the N-terminal was purchased from Biomatik Corporation (Ontario, Canada). NHS-rhodamine mixed isomers and N-ε-maleimidocaproic acid hydrazide trifluoroacetic acid salt (EMCH) were acquired from Thermo Fisher Scientific (Waltham, USA). Cyanine 5.5 hydroxysuccinimidyl ester (NHS-Cy5.5) was obtained from Lumiprobe GmbH (Hannover, Germany). Doxorubicin hydrochloride salt was purchased from LC laboratories (Woburn, Canada). The water used for the preparation of solutions was "ultrapure" water (milliQ-grade, 0.06 μS cm^{-1}), produced with the Millipore Milli-Q purification system (MA, USA).

All the materials for cell culture were obtained from Sigma-Aldrich (St. Louis, USA), including Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), penicillin, streptomycin, L-glutamine and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). HepG2 cells, derived from a human hepatocellular carcinoma (LGC Standards S.r.l., Sesto San Giovanni, MI, Italy), were used. These cells were also stably transfected with a plasmid vector carrying the wild-type SERPINB3 human gene (HepG2/SERPINB3) or with the plasmid vector alone (HepG2/empty vector) (pcDNA3.1D/V5-His-TOPO, Invitrogen Life Technologies, NY, USA), as previously reported (Quarta et al., 2010).

2.1. Pullulan oxidation

Pullulan was oxidized at 10, 20 and 40% according to the method reported in literature (Bruneel and Schacht, 1993) using a $NaIO_4$/GPU molar ratio of 0.1:1 and 0.2:1 and 0.4:1. Briefly, 200 mg of pullulan (1.23 mmol glucose units) were dissolved in 20 mL of distilled water and added of 26 mg (0.12 mmol), 53 mg (0.25 mmol) and 106 mg (0.49 mmol) of $NaIO_4$ for PullOx 10%, PullOx 20% and PullOx 40%, respectively. The solutions were kept in the dark under stirring for one hour and then added of 1.5 fold molar excess of mannitol respect to periodate to quench the oxidation reaction. The reaction mixtures were stirred for 2 h and then the by-products were eliminated by ultrafiltration using an Amicon system operated with a 10 kDa MWCO membrane (Merck Millipore, Darmstadt, Germany). The collected ultrafiltered solutions were lyophilized to obtain the oxidized pullulan derivatives as white powder (yields in the range of 92–99% w/w) that were analysed by potentiometric titration to assess the percentage of oxidation (Scomparin et al., 2011).

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