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Polymer−Drug Conjugates for Combination Anticancer Therapy: Investigating the Mechanism of Action

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We developed a family of polymer−drug conjugates carrying the combination of the anticancer agent epirubicin (EPI) and nitric oxide (NO). EPI-PEG-$(NO)_8$, carrying the highest content of NO, displayed greater activity in Caco-2 cells while it decreased toxicity against endothelium cells and cardiomyocytes with respect to free EPI. FACS and confocal microscopy confirmed conjugates internalization. Light scattering showed formation of micelle whose size correlated with internalization rate. EPI-PEG-$(NO)_8$ showed increased bioavailability in mice compared to free EPI.

Introduction

In the last decades, polymer−drug conjugates have attracted increasing attention as novel "nanomedicines" for cancer treatment.[1,2] Polymers carrying established anticancer agents such as doxorubicin and paclitaxel have entered clinical trials,[1,3] and some are expected to reach the market in the near future.[4] The advantages of these nanosized systems are now well established: (i) tumor selectivity thanks to the hyperpermeability of tumor vasculature, a phenomenon defined enhanced permeability and retention (EPR[a]) effect,[5] (ii) decreased toxicity compared to the free drug,[3] (iii) increased drug solubility,[6] and (iv) bypass of P-glycoprotein-mediated drug resistance.

Very recently, polymers carrying a combination of different therapeutic agents within a single polymeric chain have showed an improved therapeutic profile.[7−9] We have described a PEG conjugate carrying the combination of an anticancer agent, EPI, and eight NO releasing molecules.[8] EPI is currently used for the treatment of several cancers, including breast, liver, and colon cancers,[10] but its use is limited by severe cardiotoxicity.[11] The combination of NO and EPI on the same carrier presents two advantages: (i) NO has been shown to protect cardiomyocytes against doxorubicin-induced apoptosis,[12] thus, preventing anthracycline-induced cardiomyopathy, (ii) NO enhances the antitumor activity of anthracycline in vitro.[13] Polymer-conjugation of EPI and NO to the same carrier ensures that the two molecules will undergo the same body distribution, thus maximizing the anticancer effects at the tumor site while decreasing cardiotoxicity. Our initial studies confirmed that the conjugate EPI-PEG-$(NO)_8$ was able to induce a higher degree of apoptosis in Caco-2 cells to reduce EPI toxicity in embryonic rat heart-derived myoblast (H9c2) and showed a 95% tumor reduction in Caco-2 and SKOV-2 tumor-bearing mice.[8,14]

Here, we systematically investigate the correlations between chemical structure and biological behavior of EPI-PEG-$(NO)_8$ by thoroughly characterizing this conjugate and by comparing it with similar conjugates having different NO content. The aim of the present study is 2-fold: to describe the complete synthesis and characterization of this novel class of therapeutic agents and then we aim to establish correlations between the structure and the biological activity of these PEG conjugates by dissecting their mechanism of action in vitro and analyzing their pharmacokinetic in vivo. Three conjugates were prepared: EPI-PEG-$(COOH)_4$ (**1**), EPI-PEG-$(NO)_4$ (**2**), and EPI-PEG-$(NO)_8$ (**3**) bearing 0, 4, or 8 NO releasing molecules, respectively (Figure 1). The increased

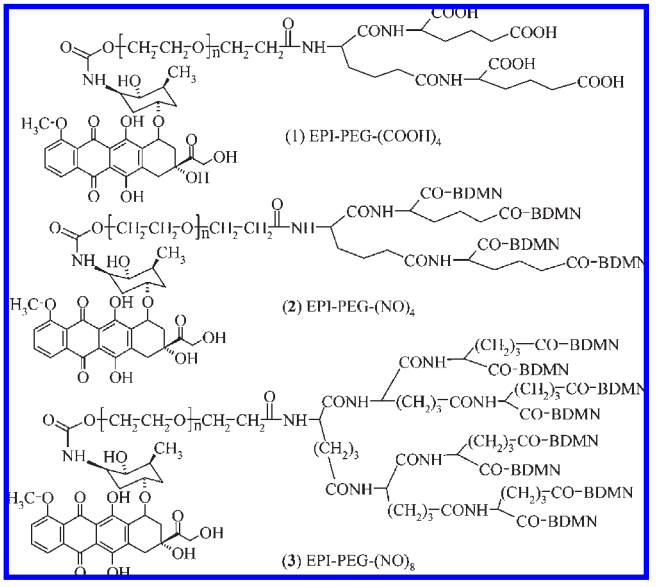

Figure 1. Chemical structure of conjugates **1**, **2**, and **3**.

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[a] Abbreviations: EPI, epirubicin; NO, nitric oxide; EPR, enhanced permeability and retention; AD, amino adipic acid; BDMN, butandiol mononitrate.

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loading of NO was achieved by synthesizing a dendrimeric structure at the polymer end chain as previously described.[15,16] The conjugates were characterized in respect of the total and free drug contents and of their stability in different environments (aqueous buffer solutions and plasma), and their biological behavior was assessed in vitro and in vivo.

Results and Discussion

Synthesis, Characterization, and Stability of the Conjugates. Products **1**, **2**, and **3** were all synthesized starting from the heterobifunctional HO-PEG-COOH (3.4 kDa). The overall yields of **1**, **2**, and **3** were 65.2, 53.4, and 45.1%, respectively. The loading of total EPI was good (between 8.6 and 10.2% w/w), and free EPI content was always less than 1.5% w/w of the total EPI (Table 1). MALDI-TOF mass spectrometry showed a broad peak centered at 4018, 4607, and 5465 m/z for **1**, **2**, and **3**, respectively (Figure A−C in Supporting Information). The broadness of the peaks can be attributed to both polymer polydispersity and the presence of isomers with different degree of BDMN coupling. The increase in the molecular weights of the conjugates is consistent with the expected higher degree of branching and subsequent BDMN content with **3** > **2** > **1**. To ensure selective tumor accumulation and maximize the therapeutic benefit, conjugates stability in biological fluids prior to arrival to the target site is an essential requirement. The conjugates were stable in all the conditions tested (buffers pH 7.4 and 5, mimicking different biological environments, and in mouse plasma), with only minimal drug release (< 5%) occurring after 24 h incubation (Table A in Supporting Information).

Evaluation of NO Release in Vitro. To assess release of NO from **2** and **3**, these conjugates were incubated with Caco-2, H9c2, and HUVEC cells. In vitro evaluation allowed quantification of NO content and assessment of NO release rate. NO release from **2** and **3** suggested that the cells were able to metabolize the conjugates. As expected, NO was not release in the case of free EPI or **1**. This confirmed that the increased levels of NO observed with **2** and **3** were the result of NO release from the conjugates rather than due to stimulation of other metabolic pathways. In addition, the absence of NO release from the conjugates after incubation without cells indicated that the NO release was cell-dependent. The release of NO correlated well with the relative theoretical NO content in the conjugates (NO release in **3** > **2** > **1**) (Figure D in Supporting Information). The NO release half-lives for conjugate **3** were in the range of 2.5−2.8 h, in all three different cell lines, which indicates that NO and EPI are released sequentially with NO having a faster release rate than EPI. This should constitute an advantage because in vitro studies have shown that NO enhances the EPI antitumor activity only when cells are pre-exposed to NO and to EPI later.[13] In vivo studies showed that **3** had superior anticancer activity than EPI and PEG-EPI, which suggest that the release pattern observed in vitro might also be advantageous in vivo. However, detailed studies are needed to clarify the exact release pattern in vivo and to determine the amount of conjugates cleared intact into urine.[14]

Evaluation of Anticancer Activity in Vitro. The anticancer activity of **1**, **2**, **3**, and EPI was investigated in vitro against a human colon carcinoma cell line (Caco-2). The advantages of polymer−drug conjugates compared to the parent free drug are normally best seen in vivo, where EPR effect-mediated selective tumor accumulation can occur. Indeed, because polymer−drug conjugates rely on cell activation, they are normally less active than the free drug in vitro.[17] However, in vitro studies are still very important to determine the relative activity of different conjugates and to investigate the mechanism of action at cellular level. All conjugates induced concentration dependent apoptosis in Caco-2 cells (Figure 2). The conjugates **2** and **3** induced higher levels of apoptosis than the NO free conjugate **1**. In the case of **3**, cell apoptosis was even higher than that induced by free EPI ($n = 8$, $P < 0.05$). mPEG-(BDMN)$_8$, a conjugate bearing the maximum NO loading as for **3** but no EPI, was synthesized starting from mPEG-COOH and exploiting the same chemical route of **3**. The conjugate was tested in Caco-2 cells as control. No cytotoxicity was observed at all concentrations tested (IC_{50} > 71 μg/mL conjugate equiv). The protective effect of NO against EPI-induced apoptosis was investigated in normal cells. H9c2 and HUVEC cells were exposed to **1**, **2**, and **3** for 48 h at a concentration of 10 μM EPI-equiv. Interestingly, while able to induce apoptosis in Caco-2 cells, the conjugates carrying the combination of EPI and NO (**2** and **3**) protected endothelial cells and cardiomyocytes (Figure 2) from EPI-induced cell death. Therefore, conjugates **2** and **3** displayed an improved tolerability profile in vitro compared to standard EPI or **1**. These results prompted us to carry out more in-depth studies to better understand the different behavior of **2** and **3**. Further biological assessment to compare the most promising conjugate **3** with that without NO (**1**) was performed.

Table 1. Physicochemical Characterization of Conjugates **1**, **2**, and **3**[a]

sample	total EPI (% of w/w)	free EPI/total EPI (% of w/w)	dn/dc (mL/g)	M_w (Da)	N_{Aggr}	R_g (nm)
1	10.19	1.41	0.143	4018	23	45
2	9.30	1.32				
3	8.58	1.20	0.145	5465	10	20

[a] N_{Aggr} = average aggregation number; R_g = radius of gyration.

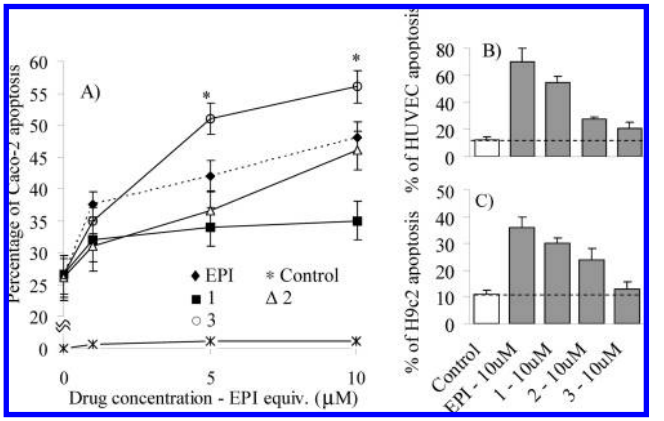

Figure 2. Anticancer activity of **1**, **2**, **3**, and EPI in Caco-2 (A), HUVEC (B), and H9c2 (C) cells. Data are expressed as mean ± SD ($n = 8$). *$P < 0.05$ vs all groups.

Conjugate−Lipids Interactions Studies. The biological activity of polymer−drug conjugates relies on endocytic uptake followed by drug release, however, some studies suggested that conjugates−membrane interactions may at least partially contribute to their biological activity. Here, interactions of **1** and **3** with a mixture of lipid that reflected the composition of biological membranes were assessed by

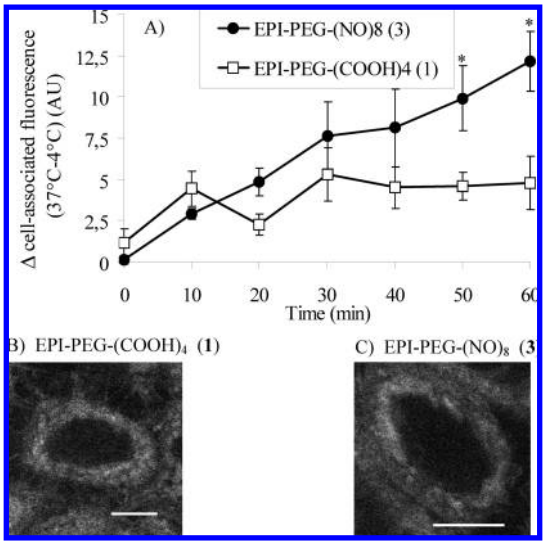

Figure 3. Cellular uptake of **1** and **3** by Caco-2 cells. (A) Internalization of conjugates: □ for **1** and ● for **3** (data expressed as mean ± SEM (n = 4); *P < 0.05 vs **1**). (B,C) Confocal microscopy pictures of cells after 1 h incubation with **1** and **3**; size bar = 10 μm.

surface pressure measurements at the air/water interface using a Langmuir trough. Neither **3** nor **1** interacted with the lipid monolayer (Figure E in Supporting Information), suggesting that it is unlikely that the conjugates exert their toxicity via interaction with the plasma membrane.

Cellular Uptake of 1 and 3. It was hypothesized that an increased cellular uptake might contribute to the increased activity of the combination conjugates (**2**, **3**) observed in Caco-2 cells. Thus, the cellular uptake of conjugates **1** and **3** was quantified by flow cytometry. The study was carried out at 37 °C and at 4 °C (the latter to account for cell binding to the membrane). Both conjugates were internalized by Caco-2 cells (Figure 3). Interestingly, the degree of internalization was more marked for **3**, which may contribute to its increased activity. Confocal microscopy studies were carried out in parallel (Figure 3). Internalization of the conjugates was observed. Indeed, both conjugates localized in vesicular structures, with a more marked accumulation in the perinuclear region. Interestingly, no membrane labeling was seen. This observation is in agreement with the surface pressure studies in model membranes, which showed no conjugates−membranes interaction.

Aggregation Properties of Conjugates in Solution. To further understand the FACS data, the aggregation properties of the conjugates were investigated by light scattering analysis. It is well-known that from a MALS experiment in off-line mode two important parameters can be obtained: the weight-average molecular weight and the z-average of the root-mean squares radius $\langle s^2 \rangle z^{1/2}$, in short radius of gyration (R_g). Conjugate **1** clearly aggregated more than **3**, with the average aggregation number (N_{aggr}) being 23 and 10, respectively. As a result, also the R_g was higher for **1**. The molecular weight distribution of **1** and **3** was assessed by size exclusion chromatography−MALS method, allowing measurement of the whole molecular weight distribution of the aggregates. After a chromatographic separation with a relatively high flow rate, the aggregation extent was lower with regard to the static off-line mode. However, these results confirmed that conjugate **1** aggregated more than conjugate **3** (Table 1). Altogether, these data demonstrate that the construct architecture influences the conjugate conformation in solution and therefore may play an important role on drug activity.

Table 2. Pharmacokinetic Data of EPI and Conjugate **3**

sample	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	$AUC_{(0-\infty)}$ (μg·min/mL)	V_d (mL)	clearance (mL/min)
EPI	1.4	182.4	1.21×10^3	46.12	0.175
3	41.0	693.0	7.55×10^4	3.74	0.014

In Vivo Pharmacokinetics. The pharmacokinetic profiles of both EPI and the most promising conjugate **3** were determined in mice. A prolonged circulation time was displayed by **3** (its area under the curve value was about 7 times higher than that of EPI), see Table 2. In addition, while EPI readily distributes to several tissues (V_d = 46.12 mL), **3**'s prolonged circulation time was also reflected in a small V_d (3.74 mL). The elimination half-life of **3** is consistent with the pharmacokinetic profile desired to obtain selective tumor accumulation by the EPR effect.[5]

Conclusion

This paper presented the full synthesis, characterization, and biological behavior of novel PEG conjugates carrying EPI and NO, establishing structure−activity relationships. The combination polymers (**2** and **3**) were found to have a better biological profile in vitro compared to the polymer carrying only EPI (**1**) or the free EPI, as they were more active against cancer cells while less toxic on endothelial cells and cardiomyocytes. In vivo pharmacokinetic analysis confirmed that **3** was in line with what was desired to obtain selective tumor accumulation. These results prompt further in vivo studies to fully elucidate their therapeutic potential.

Experimental Section

Synthesis of 1 and 2. To HO-PEG-COOH 3400 Da (0.25 mmol) in CH_2Cl_2, 4-nitrophenyl chloroformate (0.75 mmol), and Et_3N (0.75 mmol) were added. After 6 h, the reaction mixture was filtered and dropped into 250 mL of Et_2O. The intermediate was filtered and dried. Activation degree was 97.5% (Yield: 92.2%). To EPI·HCl (0.26 mmol) in DMF, the activated intermediate (0.24 mmol) was added. Et_3N (0.59 mmol) was added to the solution. After 12 h, 15 mL of HCl 0.1 N was added to the solution. EPI-PEG-COOH was extracted by CH_2Cl_2, and the organic phase was concentrated and dropped into EtO_2. EPI-PEG-COOH was purified by preparative RP-HPLC. Free and total EPI were determined as reported elsewhere.[15] Yield: 91.1%. To EPI-PEG-COOH (0.22 mmol) in CH_2Cl_2, DCC (0.67 mmol) and NHS (0.27 mmol) were added. After 3 h, the reaction mixture was filtered and dropped into EtO_2. EPI-PEG-NHS was filtered and dried (Yield: 97.7%). Activation degree, evaluated as reported elsewhere,[15] was 94.3%. To AD (0.64 mmol) in borate buffer 0.1 M, pH 8, EPI-PEG-NHS (0.21 mmol) was added. After 1 h, the pH of reaction mixture was brought to 3 by HCl 0.1 N and EPI-PEG-AD was extracted by CH_2Cl_2; the organic phase was concentrated and dropped into Et_2O. The intermediate was filtered and dried (Yield: 92.4%). The above reaction steps of carboxylic groups activation, by NHS/DCC, and coupling with AD, were reiterated until to obtain **1** (overall yield: 65.2%) or further to synthesize EPI-PEG-AD-$(AD)_2$-$(AD)_4$ (overall yield: 55.7%). Compound **1** (0.05 mmol) was added to a 15% (w/v) solution of BDMN in CH_2Cl_2. DDC (0.4 mmol) and HOBt (0.3 mmol) were added and the pH was brought to 8 by Et_3N. After 12 h, the product **2** was precipitated in ethyl acetate. The product was filtered, dried, and precipitated from CH_2Cl_2/EtO_2.

Synthesis of 3. The synthesis of **3** was achieved using the chemical route of **2**, starting from EPI-PEG-AD-$(AD)_2$-$(AD)_4$ (overall yield: 45.1%).

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Supporting Information Available: Chemistry, stability studies, MALDI-TOF MS, NO release, surface pressure−time plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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