

Review

Design, Synthesis and Applications of Hyaluronic Acid-Paclitaxel Bioconjugates[†]

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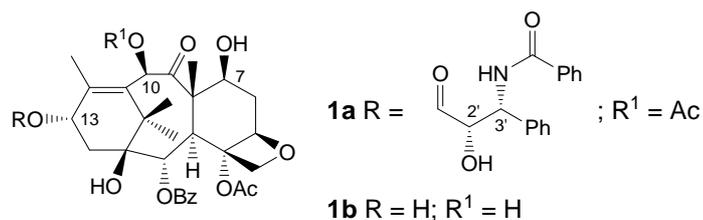
Abstract: Paclitaxel (**1a**), a well known antitumor agent adopted mainly for the treatment of breast and ovarian cancer, suffers from significant disadvantages such as low solubility, certain toxicity and specific drug-resistance of some tumor cells. To overcome these problems extensive research has been carried out. Among the various proposed strategies, the conjugation of paclitaxel (**1a**) to a biocompatible polymer, such as hyaluronic acid (HA, **2**), has also been considered. Coupling a bioactive compound to a biocompatible polymer offers, in general, many advantages such as better drug solubilization, better stabilization, specific localization and controlled release. Hereafter the design, synthesis and applications of hyaluronic acid-paclitaxel bioconjugates are reviewed. An overview of HA-paclitaxel combinations is also given.

Keywords: Paclitaxel, Hyaluronic Acid, Hyaluronic Acid-Paclitaxel Bioconjugate, Synthesis, Biological Activity.

1. Introduction

1.1. Paclitaxel

Paclitaxel (**1a**), a taxane diterpenoid isolated in 1967 from the bark of *Taxus brevifolia* (Pacific yew) [2], is a well known antitumor agent adopted mainly for the treatment of breast [3-5] and ovarian cancer [5-8]. Paclitaxel is a mitotic inhibitor which acts by interfering in the normal microtubule growth during cell division.



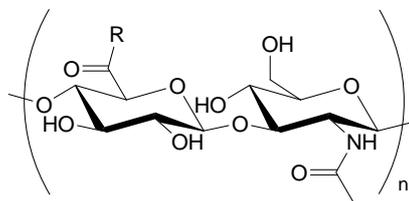
Since the beginning, the difficulty of paclitaxel availability was evident, owing to its scarcity in the bark (10 g of pure material from 1,200 Kg of bark) and to the fact that the use of this tree as the only source of compound **1a** would have rapidly caused its disappearance. In addition, owing to the complexity of its structure, paclitaxel can only be obtained in trace amounts by total synthesis. Thus, studies aimed to develop alternative ways of supply were initiated by Green, Guéritte-Voegelein and co-workers [9] and Holton [10]. These authors developed a semisynthetic route to paclitaxel starting from 10-deacetylbaccatin III (**1b**), extracted in high yield from the leaves of *Taxus baccata* L. (European yew). The Holton preparation was licensed to Bristol-Myers Squibb enabling the company to produce **1a** on a very large scale. Currently paclitaxel production employs plant cell fermentation technology [11], but the search for new semisynthetic routes or new culturing media is still in progress, due to the excellent antitumor properties of this drug.

Despite the above valuable therapeutic features, paclitaxel suffers from significant disadvantages among which low water solubility, certain toxicity (which limits the clinically administered dose) and specific drug-resistance of some tumor cells [12]. Owing to its low water solubility it is generally administered as a castor oil (Cremophor®)/EtOH solution. This type of administration requires hospitalization, since side effects such as hypersensitivity, may occur [8, 13-14]. In addition it has been reported that Cremophor® reduces the free paclitaxel fraction because of its entrapment in Cremophor® micelles [15-16].

1.2. Hyaluronic Acid

Hyaluronic acid (HA, **2**) is a glycosaminoglycan found distributed throughout the connective, epithelial and neural tissues [17]. It is one of the main components of the extracellular matrix, contributes significantly to cell proliferation and migration and is also involved in the progression of some malignant tumors where it is highly concentrated; besides, it turns out to be an important signal for activating kinase pathways [18-19] and regulating angiogenesis [20]. Moreover, since some specific HA receptors (CD44, RHAMM) are overexpressed in various malignant cell types, linking an

antitumor drug to **2** might improve targeting to cancerous cells and overcome, if the case, the problem of low drug hydrosolubility. For these reasons, HA has been linked to various antitumor drugs [21, 22].



- 2** HA R = OH
9 HA-ADH R = HNNHC(=O)(CH₂)₄C(=O)NHNH₂
11 HA-TBA R = O(Bu₄N)
13 HA-NH₂ R = NH(CH₂)₂NH₂

1.3. Paclitaxel Derivatives

To overcome the disadvantages related to paclitaxel's low water solubility and toxicity, extensive research has been carried out and various strategies have been proposed, such as for instance, changes in its formulation [8, 23-26] and the preparation of new derivatives, mainly through ester formation at the C-2' or C-7 positions [27]. In some of these derivatives, paclitaxel is linked to macromolecules, such as poly(ethyleneglycol) [28-42], *N*-(2-hydroxypropyl)methacrylamide [39, 43-47], carboxymethyl dextran [48], poly(*L*-glutamic acid) [49-74], peptides [75-76], proteins [77-79], dendrimers [37, 42, 80-81] and HA [82-87]. Joining a bioactive compound to a biocompatible polymer offers, in general, several advantages like better drug solubilization, stabilization, localization and controlled release [88-90]. For derivatives other than those quoted above, see [91-100].

In the present review we shall deal only with HA-paclitaxel bioconjugates. To the best of our knowledge, only four HA-paclitaxel bioconjugates **3-6** (Figure 1) have been described so far in the literature, differing mainly in the anchor chain between HA and paclitaxel.

Prestwich and co-workers, at Stony Brook University in New York and at the University of Utah in Salt Lake City, pioneered these studies and made a major contribution to the field [82-83]. This work was followed by the work at University of Rome "La Sapienza" of Crescenzi, Marini Bettolo and co-workers [84-86], in collaboration with Padua based Fidia Farmaceutici S.p.A., and by that of Tabrizian and co-workers at McGill University in Montreal [87]. Hereafter we wish to report on the design, synthesis and applications of HA-paclitaxel bioconjugates **3-6**, prepared by the above mentioned groups. An overview of HA-paclitaxel combinations is also given.

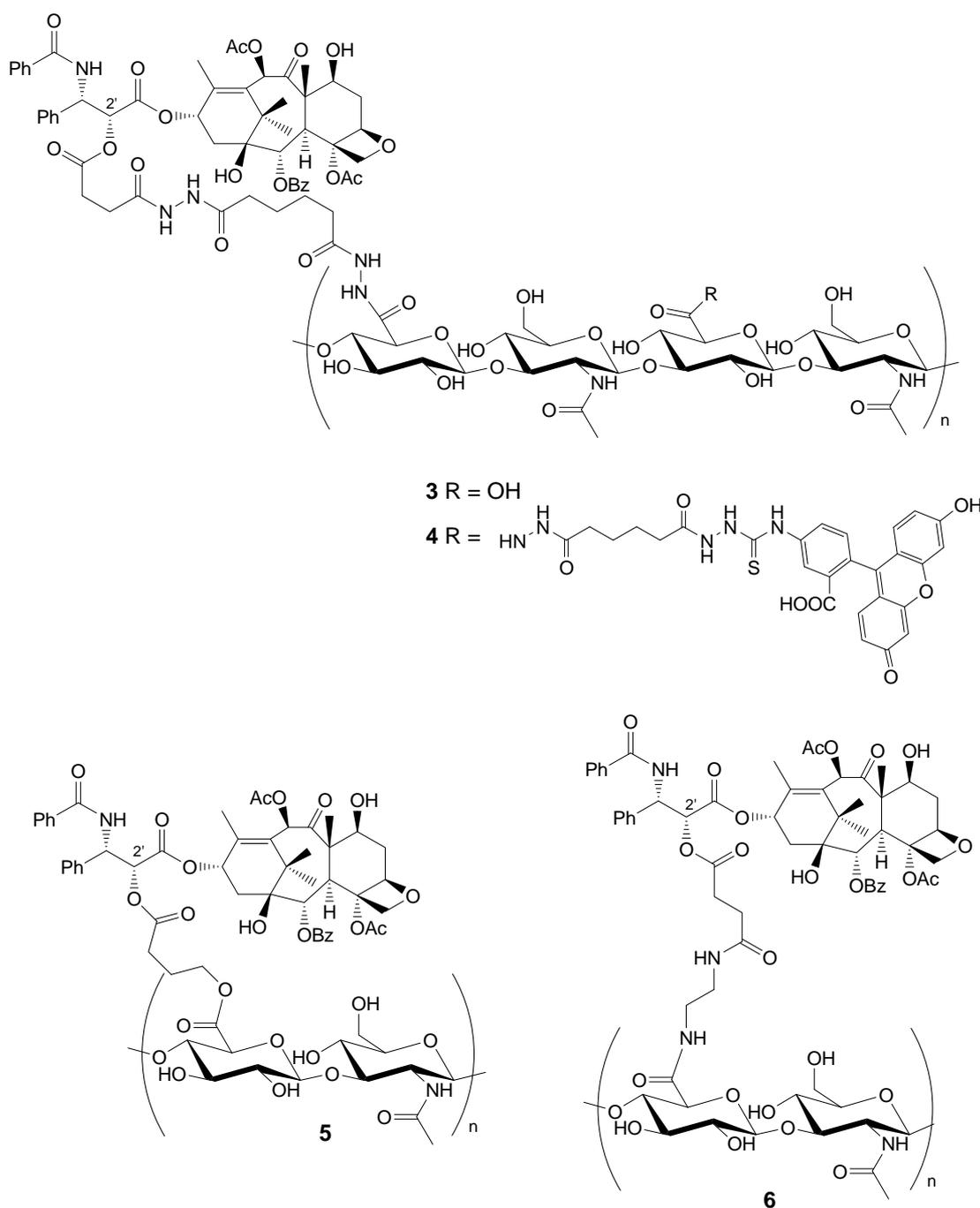
2. Synthesis and Applications of HA-paclitaxel Bioconjugates 3-4

2.1. Synthesis

Prestwich and co-workers prepared bioconjugate **3** [82-83] by the dihydrazide method [101-103]. This method was developed with the aim of obtaining under mild conditions, necessary to avoid HA degradation, a HA derivative bearing a terminal hydrazido group which would have allowed further

coupling still under mild conditions. The spacer should have eased intracellular enzyme degradation or hydrolytic cleavage [90]. Thus paclitaxel **1a** was first reacted with succinic anhydride and pyridine at room temperature for 3 days to give the 2'-hemisuccinate paclitaxel derivative **7**, that in turn was transformed, by a reaction with *N*-hydroxysuccinimido diphenyl phosphate, into 2'-ester **8** containing a *N*-hydroxysuccinimide moiety. Finally **8** was reacted with adipic dihydrazide modified HA **9** (HA-ADH), prepared from low molecular weight HA **2** and adipic dihydrazide [101-103] at pH 6.5 [104] in a 3mM phosphate buffer and DMF mixture (Scheme 1).

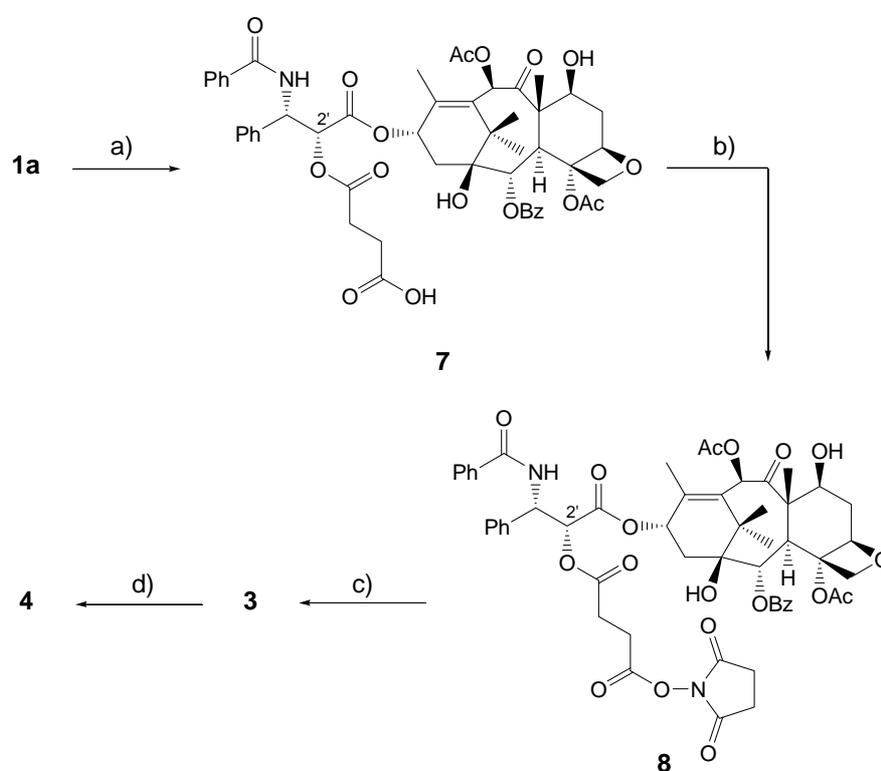
Figure 1. HA-paclitaxel Bioconjugates **3-6**.



The reasons for using low molecular weight HA were: to monitor the biopolymer loading by NMR; to have a readily injectable non viscous solution; besides, once in the plasma, low molecular weight HA is taken up quickly by cells without further degradation and can be expelled from the body *via* kidneys. Coupling paclitaxel derivative **8** with HA-ADH **9**, gave bioconjugate **3** after dialysis and lyophilization. By varying the **8/9** ratio several bioconjugates of type **3** were prepared, whose hydrosolubility depends on ADH and paclitaxel 2'-hemisuccinate loading.

Evaluation of bioconjugate **3** ability to bind to tumor cells and its subsequent internalization by cells, was achieved by converting it with fluorescein isothiocyanate (FITC) in DMF into the fluorescently labeled HA-paclitaxel derivative **4** [83].

Scheme 1. Preparation of Bioconjugate **3**.



a) Succinic anhydride, CH_2Cl_2 , pyridine, r.t., 3 d; b) *N*-hydroxysuccinimidobiphenyl phosphate, MeCN, Et_3N , r.t., 6 h; c) HA-ADH **9**, DMF/ H_2O 2:1, r.t., 24 h, pH 6.5 [82-83] or pH 8.5 [105]; d) fluorescein isothiocyanate, DMF, r.t., 12 h.

2.2. *In Vitro* Antitumor Activity of Bioconjugates **3-4**

Bioconjugate **3** showed effective *in vitro* cytotoxicity against HCT-116 (colon tumors), SK-OV-3 (ovarian cancer) and HBL-100 (breast cancer) human cell lines [82-83]. In contrast, no cytotoxicity was observed against NIH 3-T-3 (nontransformed mouse fibroblast) cell lines. Selective toxicity was attributed to receptor-mediated binding and uptake of HA-paclitaxel bioconjugate owing to overexpression of CD44 receptors by the above human cell lines. Selective targeting due to the receptor characteristic was then confirmed by the possibility of blocking bioconjugate uptake and

toxicity with a 100-fold excess of high molecular weight HA and with anti-CD44 mAb; different results were obtained with a 100-fold excess of chondroitin sulfate (a sulfated glycosaminoglycan).

Paclitaxel (**1a**) release from bioconjugate **3** was evaluated in various media. From these experiments it was found that cleavage occurs at C-2' ester function, owing to the greater stability of the hydrazone linkage over the ester bond. Thus only free **1a** is released. It was also observed that release was dramatically accelerated in the presence of hydrolytic enzymes. Prestwich and co-workers showed also that cytotoxicity of bioconjugate **3** depends on both HA (**2**) modification and paclitaxel (**1a**) loading: high loading, in fact, lowers solubility and causes modification in the HA structure masking the HA receptor recognition elements resulting in a cytotoxicity decrease.

In vitro cytotoxicity of **3** against the CD44(+) human ovarian tumor cell lines SK-OV-3ip and NMP-1 was also recently evaluated by Klostergaard and co-workers [105] at the University of Texas in Houston. Their results turned out to be in good agreement with those previously obtained by Prestwich and co-workers [82-83], especially as far as “cell targeting” ability is concerned. Klostergaard and co-workers have shown, in fact, that the ability of **3** to reduce cell survival was inhibited by preblocking the HA binding sites with HA **2** (≈ 40 kDa).

2.3. *In Vivo* Antitumor Activity of Bioconjugate **3**

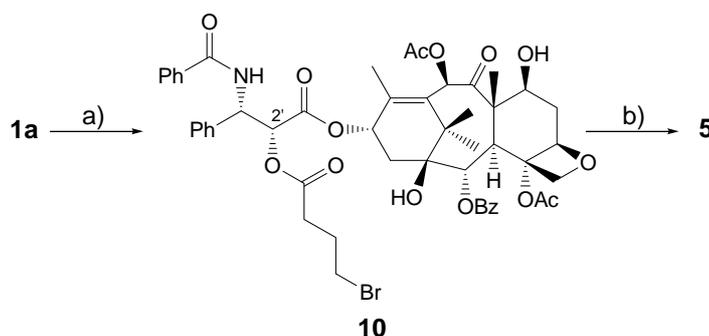
Better *in vivo* antitumor efficacy and lower toxicity against CD44(+) human ovarian carcinoma xenografts NMP-1 and SKOV-3ip, as compared to free paclitaxel, was also demonstrated by the same authors for bioconjugate **3** [105].

3. Synthesis and Applications of HA-paclitaxel Bioconjugate **5**

3.1. Synthesis

The approach chosen by Crescenzi, Marini Bettolo and co-workers, in collaboration with FIDIA Farmaceutici S.p.A., a pharmaceutical company involved for many years in the production and derivatization of HA, is quite simple. Paclitaxel **1a** was joined to HA by means of a spacer linked to both paclitaxel **1a** and HA **2** *via* ester functions (Scheme 2) [84-86].

Scheme 2. Preparation of Bioconjugate **5**.



a) 4-Bromobutanoic acid, EDC, DMAP, CH_2Cl_2 , r.t.; b) HA-TBA **11**, NMP, r.t.

Thus, paclitaxel was treated at room temperature with 4-bromobutanoic acid in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and *N,N*-dimethylpyridine-4-amine (DMAP) to cleanly give in 78 % yield paclitaxel 2'-(4-bromobutanoate) (**10**, Scheme 2). The authors confirmed the formation of a 2'-*O*-substituted paclitaxel derivative by the characteristic downfield shifts in the ¹H- and ¹³C-NMR of the signals of H-C(2') from δ (H) 4.77 [91] to 5.50 and of C(2') from δ (C) 73.2 [91] to 74.2, respectively.

Compound **10** was then dissolved in 1-methylpyrrolidin-2-one (NMP) and treated for 7 days at room temperature with HA-TBA **11** (Mw = 185 kDa; **10/11** 1:4) to give **5**. The ratio of reactants chosen was a good compromise to give hydrosoluble bioconjugate. Bioconjugate **5** was isolated from the reaction mixture after EtOH/NaCl precipitation, extensive dialysis (cut off *ca.* 2 kDa) against distilled water and finally freeze-dried.

A *ca.* 25% substitution degree was deduced for **5** by comparing its UV absorbance in EtOH/H₂O 7:3 with a UV-absorption calibration plot of paclitaxel **1a** and HA-Na. Compound **5** was hydrosoluble. As for the bioconjugate **3** paclitaxel loading can be varied by changing the **10/11** ratio.

3.2. *In Vitro* Antitumor Activity of Bioconjugate **5**

The *in vitro* antitumor activity of a hydrosoluble bioconjugate **5** with a 20% wt/wt carboxyl esterification, was tested in respect to bladder cancer cells by Rosato and co-workers at Padua University [85, 106]. The *in vitro* **5** inhibitory activity on the growth of RT-4 and RT-112/84 bladder cancer cells was much stronger than that of paclitaxel (**1a**). Furthermore, the powerful increase in efficiency of **5** in respect to **1a** was attributed, as in the case of bioconjugate **3**, to the active intracellular uptake mediated by specific HA receptors (CD44) overexpressed on the tumor cell surface and a subsequent hydrolytic release of the active drug in the intracellular medium only. To prove whether the CD44 HA receptor could directly interact with **5**, the CD44 expression was also analyzed at different times after RT-4 and RT112/84 cells were incubated with either the bioconjugate **5** or HA **2**. In both cases the authors noted a similar striking up-regulation in the CD44 expression showing therefore a direct interaction of conjugated HA with the receptor. The stability of **5** was tested in human urine and no bioconjugate degradation or paclitaxel release was observed 6 hours after incubation (pH 6.5).

3.3. *In Vivo* Antitumor Activity of Bioconjugate **5**

Rosato and co-workers also studied [106] the *in vivo* antitumor therapeutic activity of bioconjugate **5** by inoculating subcutaneously RT-112/84 TCC bladder tumor cells in mice. A comparison between the antitumor activity of paclitaxel (**1a**) and bioconjugate **5** showed that the efficacy of **1a** and **5** is equal though the former has a slightly stronger cytoreductive activity.

A pharmacokinetic analysis of **5** was also performed in order to exclude the presence of paclitaxel **1a** in the blood after administration of bioconjugate **5** to rat bladder. According to the authors, although the stability of **5** had already been tested *in vitro*, it might be possible that release of paclitaxel takes place *in vivo* after bladder instillation. The experiments confirmed that paclitaxel concentration in the blood was negligible, since after administration it remained entirely confined in

the bladder. Mice were also treated locally with bioconjugate **5** and paclitaxel to determine whether **5** was well tolerated by the urothelial mucosa. From this study it emerged that bioconjugate **5** is very well tolerated and induces only slight morphologic changes in the urothelial epithelium, while paclitaxel produces notable toxic effects on the bladder.

In order to establish its potential therapeutic applications and to evaluate its biodistribution after intravenous, intravesical, oral or intraperitoneal administration in healthy mice, Meléndez-Alafort and co-workers [107] at the University of Padua, labeled bioconjugate **5** with ^{99m}Tc by treating it with a ^{99m}Tc -pertechnetate solution, SnCl_2 and sodium gluconate. The solution was stirred and incubated at 65°C for 90 min. The radiopharmaceutical was purified by size exclusion chromatography and the radiochemical purity of the labeled bioconjugate **5** prepared in this way was 100%. The ^{99m}Tc labeled bioconjugate **5** was stable for 6 h at 37°C in a phosphate buffer.

From biodistribution studies it emerged that the animals injected intravenously with the ^{99m}Tc labeled bioconjugate **5** showed a rapid and high liver and spleen uptake, while those administered intraperitoneally, intravesically and orally showed that it remained at the administration site. Therefore, according to Meléndez-Alafort and co-workers, bioconjugate **5** should be administered intravenously for liver metastasis therapy, orally or intravesically for local treatment of bladder and superficial cancers and intraperitoneally for ovarian cancer or other tumors in the peritoneal cavity.

4. Synthesis and Applications of HA-paclitaxel Bioconjugate **6** and Biomaterial **14**

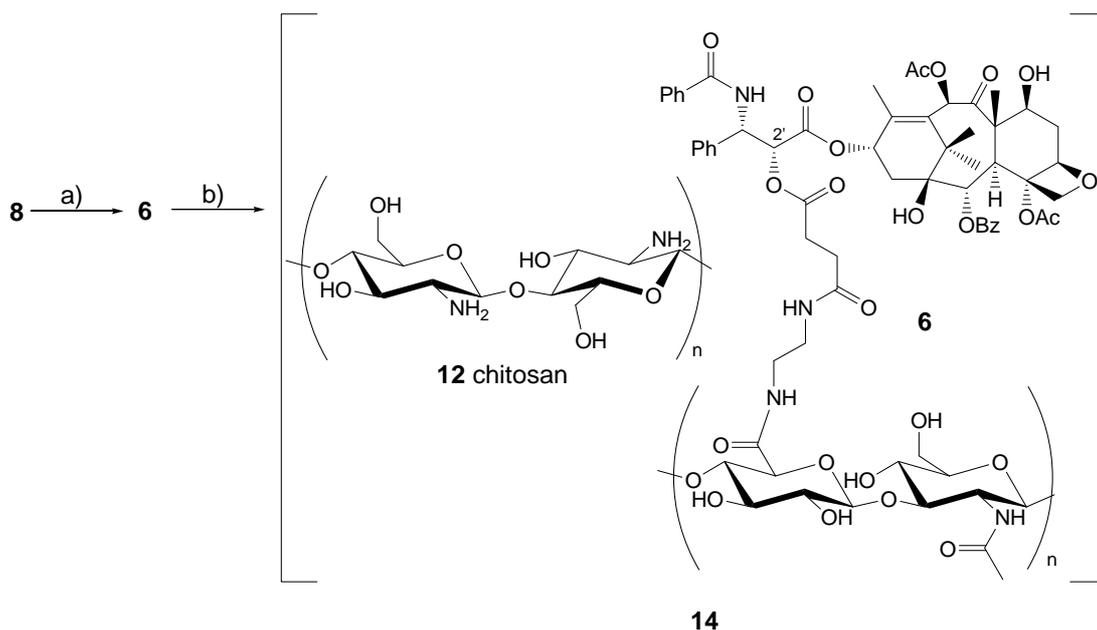
4.1. Synthesis

In the field of drug delivery, great attention has been recently devoted to the possibility of incorporating bioactive molecules into polymeric matrices [108-113]. An approach developed some years ago based on alternate deposition of polyanion and polycation layers leading to polyelectrolyte multilayer (PEM) films, constructed by the layer-by-layer (LbL) technique, can be used to build up polymeric matrices in a controlled manner. PEMs may present some advantages in drug delivery such as the possibility of including several drugs. It is, therefore, suitable for complex releasing pharmacokinetics. Tabrizian and co-workers [87] reported on the assembling of HA-paclitaxel bioconjugate **6** (Figure 1), previously described by Prestwich and co-workers [82] in a PEM system with chitosan **12** (CH) to give biomaterial **14**.

The preparation of biomaterial **14** started from known **8** [82] (Section 2.1 and Scheme 1). The latter was reacted with **13** (see section 1.2), prepared in turn treating HA (**2**, Mw = 0.5 MDa) with ethylenediamine hydrochloride (EDA) and EDC in a buffered medium (pH 5) at r.t. for 12 h, purifying the reaction mixture by dialysis against water and freeze-drying the resulting aqueous solution.

Coupling of paclitaxel derivative **8** with **13** was performed at room temperature for 24 h in a DMF and phosphate buffer (pH 6.5). After that time the crude compound was purified by dialysis to give two bioconjugates **6** with 3 and 6 mol % paclitaxel loading respectively; only the former, however, exhibited the hydrosolubility necessary for the PEM preparation.

Scheme 3. Preparation of Biomaterial 14.



a) **13**, DMF, pH 6.5, r.t., 24 h; b) polyelectrolyte multilayer construction with polyethyleneimine, HA **2**, HA-paclitaxel **6**, chitosan (**12**).

The final multilayer was obtained by consecutive adsorption of oppositely charged polyelectrolytes [CH, HA-paclitaxel **6** (3 mol %)]. Paclitaxel **1a** release studies were conducted on 10 bilayers of CH/HA-paclitaxel (CH/HA-paclitaxel)₁₀. The latter were kept in contact with water which was then removed at different times. By measuring the UV absorbance of released compound **1a**, it was found that the half-life time of **1a** was \approx 3 h. PEM constructed with rhodamine-labeled chitosan (CH-Rho/HA-paclitaxel)₁₀ were subjected to the same release test; contact water UV spectra showed only the paclitaxel absorbance peak, while that of rhodamine was lacking, proving in this way the multilayer stability during release experiments.

4.2. In Vitro Antitumor Activity of Biomaterial 14

In vitro activity of biomaterial **14** was evaluated by means of a cell viability assay. While cells cultured onto (CH/HA-paclitaxel)₁₀ showed a 95% reduction in viability after 4 days, those cultured onto (CH/HA)₁₀ showed no reduction in viability [87].

5. HA-paclitaxel Combinations

Although this review is mainly focused on the HA-paclitaxel bioconjugates, for the sake of completeness, we wish to present hereafter a small section on HA-paclitaxel combinations, *i.e.* species in which no covalent links between HA (**2**) and paclitaxel (**1a**) are present, and on their bioactivity.

5.1. Paclitaxel-loaded Crosslinked HA Films for the Prevention of Postsurgical Adhesions

Burt and co-workers at the University of British Columbia [114] investigated the use of paclitaxel (**1a**) as an inhibitor of postsurgical adhesion by loading it into a biocompatible, mucoadhesive film of crosslinked HA to be applied to abraded tissues in order to release the drug over 2-3 days. Since **1a** is as a powerful wound healing inhibitor, its permanence on the wound for a limited time might reduce the formation of postsurgical adhesions. After several optimization studies, HA films crosslinked with 2 mM EDAC and 10% glycerol were found to possess suitable flexibility, elasticity and dissolution properties. In these films paclitaxel was present as a solid dispersion. According to Burt and co-workers a possible mechanism for drug release involves the uptake of water, the swelling of the crosslinked HA matrix and the dissolution of dispersed paclitaxel.

An *in vivo* comparison between the administration of paclitaxel on the abrasion of the rat cecal side wall by repeated intraperitoneal injections in a 1:1 Cremophor EL®:ethanol formulation and by loading it into a crosslinked HA film showed similar, though incomplete, inhibition of adhesion formations in rats.

5.2. *In vivo* Inhibition of Mice Lewis Lung Carcinoma and U14 Cervical Tumor By Combination of Paclitaxel and HA.

Yuan and co-workers [115] at Tianjin University have investigated the effects of combined administration of HA (**2**) with Cremophor® solutions of paclitaxel (**1a**) on the control of Lewis lung carcinoma (LLC) migration and ascites formation of U14 cervical tumor. *In vivo* studies on mice showed that the combined use of HA and paclitaxel is more effective in inhibiting metastasis of LLC and U14 than HA or paclitaxel alone. Thus, Yuan and co-workers hypothesized that the synergic behavior of paclitaxel and HA might be due to an increased host immunity. The increase in the expression of vitamin D₃ binding protein (DBP), a macrophage stimulating activator, may be a crucial factor in inhibiting the activity of tumor cells. Yuan and co-workers [115] found that DBP expression was increased by administering the paclitaxel-HA combination. The administration of HA or paclitaxel alone did not have the same effects.

5.3. Polyelectrolyte Multilayers Films Incorporating Paclitaxel

As outlined in Section 4, PEMs have recently become an appropriate substrate coating able to incorporate biological factors for example peptides, proteins, hormones, growth factors or drugs [116-121]. The final aim of such approaches is that of controlling the rate and selectivity of cellular adhesion.

In their studies at Louis Pasteur University in Strasbourg Vodouhê, Lavallo and co-workers [122] designed a polylysine/hyaluronic acid (PLL/HA) based multilayers surface coating which acts as a reservoir for paclitaxel without the need of chemical modifications both on the PEM and on the drug. The amount of compound **1a** embedded in PLL/HA films could be finely tuned. The authors tested the viability of HT29 cell line seeded on (PLL/HA)₃₀ film. Unfortunately these *in vitro* studies showed these films did not adhere the cells. The HT29 cellular adhesion occurred when the film surface was

modified by adding a poly(sodium 4-styrene sulfonate) (PSS) layer on the top of PLL/HA films. As shown previously by Chan and co-workers [123], a sulfonate group of PSS chains adsorbed on the surface not only is able to promote cellular adhesion, but also allows modulation of the accessibility of HT29 cells to paclitaxel in terms of delay and/or kinetics by varying its composition. Paclitaxel activity remained constant after embedding in the polyelectrolyte multilayers and cellular viability could be reduced of about 80% 96 h after seeding.

6. Conclusions

HA-paclitaxel hydrosoluble bioconjugates appear promising in cancer therapy. Their cytotoxicity against various cancer cell lines is, in fact, comparable to that of free paclitaxel (**1a**) and systemic toxicity reduced owing to selective targeting of cancer cells due to HA CD44 receptors overexpression. Besides, as illustrated in the Introduction, the problems connected with the administration of paclitaxel in a castor oil (Cremophor®)/EtOH solution can be avoided. From biodistribution studies it appears recommendable a different way of administration depending on tumor localization. The preparation of nanobiomaterials incorporating HA-paclitaxel bioconjugates offers a further solution for the delivery of this valuable drug. Finally very interesting results were obtained with HA-paclitaxel combinations both for cancer chemotherapy and for better wound healing and prevention of postsurgical adhesion.

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References and Notes

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