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Short communication

Synthesis and preliminary *in vitro* biological evaluation of 7α-testosterone–chlorambucil hybrid designed for the treatment of prostate cancer

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ABSTRACT

The synthesis of 7α-testosterone–chlorambucil hybrid is reported. This compound is made from testosterone in a 6 step reaction sequence and with 23% overall yield. An alternative convergent reaction sequence yielded the same hybrid through a Grubbs metathesis reaction between chlorambucil allyl ester and 7α-allyltestosterone with 35% overall yield. MTT assays showed that the hybrid is selective towards hormone-dependent prostate cancer cell line (LNCaP (AR+)) and shows similar activity than the parent drug, chlorambucil. Thus, the new hybrid shows promising potential for drug targeting of hormone-dependent prostate cancer through its capacity of delivering chlorambucil directly to the site of treatment. This could extend the use of chlorambucil to prostate cancer in the future.

1. Introduction

In Canada, prostate cancer is the most frequently diagnosed cancer in men accounting for 28% of all new cancer cases [1]. However, prostate cancer ranks third with 10% of all cancer deaths in men, after lung and colorectal cancers with 28% and 12% of all cancer deaths, respectively [1]. Nonetheless, it is a serious health problem, not only in Canada but worldwide [2].

The androgens, testosterone (T) and dihydrotestosterone (DHT) are implicated in the development and normal functions of prostate cells. They are also involved in male sexual organ growth and sexual function. Testosterone is the principal androgen in the blood while DHT is the most potent androgen in the cells [3]. In order to induce their biological effects, androgens have to bind to the androgen receptor (AR): the hormone–receptor complex binds DNA and modulates gene expression [4]. Upon androgen stimulation, the proliferation of prostate cells is increased and a malignant tumour can develop [4]. In addition, the androgen receptor level is higher in prostate cancer cells compared to normal cells [4]. Consequently, androgens are involved not only in prostate tumorigenesis, but also in hormone-dependent cancer progression, supporting the use of androgen deprivation therapy in prostate cancer patients [5]. However, one limitation is that most tumours become resistant to this type of therapy and so, additional options of treatments are required to care for the patient. This letter reports on the development of such alternate treatment for prostate cancer.

Chlorambucil is an alkylating agent of the nitrogen mustard group and is used as cytostatic drug in cancer therapy [6]. In general, alkylating agents are both mutagenic and genotoxic [7]. The alkylating agents form adducts with DNA. However, they also form adducts with RNA and protein which contribute to the overall cytotoxicity [7]. The main side effects of chlorambucil are bone marrow suppression, anaemia and weak immune system [8,9].

Recently, we reported a series of estradiol–chlorambucil hybrids as anticancer drugs for site-directed chemotherapy of breast cancer [10]. The new hybrids showed moderate to significant cytotoxic activity in hormone-dependent (MCF-7) and hormone-independent (MDA-MB-436 and MDA-MB-486) breast cancer cell lines. Unfortunately, the hybrids were not selective towards the hormone-dependent breast cancer cell line MCF-7. Despite this, we sought to apply this type of strategy to the development of testosterone–chlorambucil hybrid for the treatment of prostate cancer. Therefore, this study was undertaken to verify if that

Abbreviations: T, testosterone; DHT, dihydrotestosterone; AR, androgen receptor; AR^+, androgen receptor positive; AR^-, androgen receptor negative; T-CHL, 7α-testosterone–chlorambucil hybrid.

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particular combination could lead to a selective hybrid towards hormone-dependent prostate cancer *in vitro*.

In order to increase the potential for selectivity towards the androgen receptor, the known 7α-allyltestosterone was selected as the starting material. Position 7 is considered the site of choice as it is located midway between the two functional groups found on testosterone (ketone and hydroxyl) that interact with the AR. These functional groups should remain intact and free of steric hindrance to favour AR binding [11–13]. Furthermore, it was decided to link the testosterone moiety to chlorambucil via an ester bound. It was speculated that the ester linkage would be sufficiently stable to reach the target cells and would have the potential to be hydrolyzed within the acidic environment of the cancer, releasing the anticancer agent chlorambucil. If successful, this approach would broaden the use of chlorambucil to hormone-dependent prostate cancer.

The current manuscript describes the synthesis of 7α-testosterone–chlorambucil hybrid (**1**) (Fig. 1) using two synthetic methods. The steroid and the nitrogen-mustard alkylating parts are linked together by a *trans*-but-2-enyl tether chain. The manuscript also presents preliminary biological evaluation of the novel hybrid on two prostate cancer cell lines; LNCaP (androgen receptor positive; AR^+) and PC3 (androgen receptor negative; AR^-).

2. Results and discussion

2.1. Chemistry

Testosterone (**2**) was initially functionalized using a known three-step reaction sequence (Scheme 1). The 7α-allyltestosterone (**3**) was obtained with 48% overall yield as described earlier [11–13]. Of note, this reaction sequence can be performed with up to 63% overall yield.

Scheme 1 illustrates the synthesis of testosterone–chlorambucil hybrid (**1**) by a S_N2 type substitution reaction. First, the 7α-allyltestosterone derivative (**3**) was subjected to an olefin cross-metathesis reaction. For this purpose, derivative **3** and allyl chloride were treated with Hoveyda-Grubbs catalyst 2nd generation in dichloromethane (DCM) at reflux for 8–10 h [14,15]. This reaction yielded 7α-(4-chloro-but-2-enyl) testosterone (**4**) in 90% yield as a mixture of *cis* and *trans* isomers (1:9). Hydrolysis of the acetate was performed in the presence of aqueous hydrochloric acid (HCl) in methanol (CH_3OH) under mild reflux to yield derivative **5** (94%, *cis*:*trans*, 1:9). Then, the substitution reaction was done using the allyl chloride **5**, chlorambucil and sodium bicarbonate in a mixture of DMF and water at reflux for 9 h. The testosterone–chlorambucil hybrid (**1**) was obtained with 58% yield (*cis*:*trans*, 15:85). Through purification, the mixture was slightly enriched into the *cis* isomer. Starting from testosterone, the complete sequence of reaction uses only 6 chemical steps and gave hybrid **1** with 23% yield.

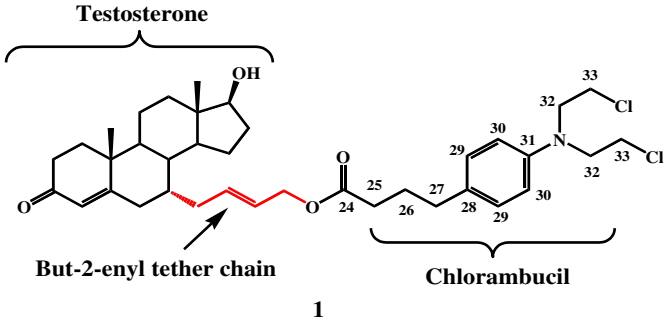

Fig. 1. Structure of 7α-testosterone–chlorambucil hybrid (**1**).

Scheme 2 illustrates an alternative synthesis of hybrid **1** by an olefin cross-metathesis reaction. Initially, derivative **3** was hydrolyzed with 10% aqueous HCl in methanol under a light reflux to yield 7α-allyltestosterone (**6**) with 95% yield. Secondly, chlorambucil was transformed into 4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyric acid allyl ester (**7**) upon treatment with oxalyl chloride followed by reaction with allyl alcohol and pyridine in dichloromethane with 95% yield. Derivatives **6** and **7** (excess) were treated with Hoveyda-Grubbs catalyst 2nd generation in DCM at reflux for 24 h. This reaction yielded the testosterone–chlorambucil hybrid (**1**) with 78% yield (*cis*:*trans*, 15:85). It is noteworthy, that the same reaction provided the dimer **8** (as a side product which was easily isolated by flash column chromatography). The latter is also an anticancer tetraalkylating agent. Of note, the dimer **8** could be easily hydrolyzed to chlorambucil which could be recycled to the allyl ester **7** and coupled to 7α-allyltestosterone **6** in order to avoid the loss of starting material. Derivative **8** was also synthesized by self condensation of **7** upon treatment with Hoveyda-Grubbs catalyst 2nd generation in DCM at reflux for 15 h with 75% yield. All compounds were fully characterized by the use of infrared (IR), nuclear magnetic resonance spectroscopy (NMR) and their respective high resolution mass analysis.

2.2. Antiproliferative activity

The second objective of the present study was to determine the cytotoxic effect of the hybrid along with controls (chlorambucil and cyproterone acetate) on both androgen-dependent (AR^+) and androgen-independent (AR^-) human prostate cancer cells. The biological activity of the compounds was evaluated *in vitro* using the MTT cell proliferation assay [16,17]. The MTT assay was performed over an incubation period of 72 h.

As shown by the MTT assays (Table 1), the new 7α-testosterone–chlorambucil hybrid (**1**) showed differential toxicity towards the two human prostate cancer cell lines used in our study (LNCaP (AR^+) and PC3 (AR^-)) compared to chlorambucil itself. Hence, hybrid (**1**) exhibited an IC_{50} of 101.0 μM for LNCaP cell line and was completely inactive towards the PC3 cell line at the maximum dose tested (160 μM, see Table 1). Chlorambucil was active on both types of cells with an IC_{50} of 124.3 μM and of 131.3 μM for, respectively, LNCaP and PC3 cell lines. Of note, despite the low activity of chlorambucil *in vitro*, it remains a very useful anticancer drug used daily in clinics. So, one should always be aware of the known discrepancies between *in vitro* and *in vivo* results [18–20]. The ability of chemosensitivity assays cannot always accurately predict the activity of a new drug *in vivo*. This study shows that the hybrid (**1**) is as active as chlorambucil *in vitro* which prelude favourably for its potential *in vivo*. Furthermore, the selectivity of hybrid (**1**) may be useful in the treatment of hormone-dependent prostate cancer reducing toxicities associated with chemotherapy. The hybrid (**1**) and chlorambucil are less cytotoxic than cyproterone acetate (CPA), a clinically used steroid-based antiandrogen. Of course, the mechanism of action of a nitrogen-mustard based drug is quite different from that of an antiandrogen.

While the affinity for the androgen receptor (AR) is an important data to take into account, and bearing in mind the relatively low activity of the final hybrid (**1**), it was decided not to measure the AR affinity at the moment.

3. Conclusion

This manuscript presents two efficient syntheses of 7α-testosterone–chlorambucil hybrid (**1**). Derivative **1** is readily available from testosterone either in a 6 step sequence with 23% overall yield or by following a more convergent 5 step sequence with 35% overall