Preparation and \textit{in vitro} cytotoxic evaluation of taxol immunoconjugates

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The taxol (paclitaxel) immunoconjugates prepared by derivatizing the taxol at two positions and linking the Mab CIBCNSH3 against EGF receptors were tested for \textit{in vitro} cytotoxicity. Succinyl derivatives of taxol at 2′-OH and 7-OH groups were prepared as per the published procedures. Both the products were purified by column chromatography. The 7-succinyl taxol and 2′-succinyl taxol immunoconjugates were prepared with CIBCNSH3, Mab against EGF receptor using a published procedure involving water soluble EDC. The unbound taxol derivatives were separated either by dialysis or concentrin separators. The drug to antibody ratio was estimated by either extracting the linked drug after hydrolysis of a conjugate or simultaneous Beer’s law. The Drug-immunoconjugates were tested for their \textit{in vitro} cytotoxic effect on EGF receptor expressing cells either A-549 or MCF-7 and HBL-100 by using MTT protocol and the IC\textsubscript{50} values were calculated. The drug to antibody ratio was 0.45:1 for 7-succinyl taxol, and 0.38:1 for 2′-succinyl taxol. The IC\textsubscript{50} value was 78 nM on A-549 cells for 7-succinyl taxol conjugate, while for 2′-succinyl taxol conjugate the IC\textsubscript{50} values were 114.8 nM and 120.3 nM on MCF-7 and HBL-100 cells respectively. Overall, as compared to the pure antibody both taxol immunocoujugates showed more cytotoxic activity.

**Keywords:** taxol immunoconjugates, succinyl taxols, Mab conjugates, drug immunoconjugates, drug targeting and anticancer conjugates

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**Introduction**

Cancer chemotherapy is based on the premise that rapidly proliferating tumour cells are more likely to be killed by cytotoxic drugs. Unfortunately, the difference in activity of current drugs against tumour tissues in comparison to healthy tissues is relatively small. The amount of a drug required to achieve a clinically effective level of concentration often causes severe damage to the actively propagating non-malignant cells, such as the cells of gastrointestinal tract and bone marrow, thereby resulting in a variety of undesirable side effects\textsuperscript{5}. The best way of circumventing these problems is to deliver the drug in a manner that is preferentially localized at the desired site of action or predominantly attacks the diseased cells\textsuperscript{2}.

The designs of many novel drug delivery systems are proposed to deliver the pharmacological agent to its site of action at therapeutically optimal dosage regimen\textsuperscript{5}. This site specific or targeted delivery combined with delivery at an optimal rate would not only improve the efficacy of drug but would also reduce the possible unwanted side effects of drug and thus improving the therapeutic index\textsuperscript{4}.

The field of drug targeting had its inception in 1906 when Paul Ehrlich proposed that chemotherapeutic drug could be site specifically delivered to the target tissue with the use of antibodies derived from the mammalian immune system\textsuperscript{5}. The vision remained dormant until Mathe and co-workers began to examine the concept of covalently attaching methotrexate to antibody\textsuperscript{6}. In the mid 1970s, Kohler and Milstein reported the successful construction of a hybridoma cell, which produced a monoclonal antibody (Mab)\textsuperscript{7}.

The discovery of antigens that are particularly over expressed on the surface of cancer cells, suggests that by using certain antibodies to selectively “Mark” tumour cells, malignant tissues could be distinguished from normal tissues. Monoclonal antibodies (Mabs), which have shown high binding specificity for tumour-specific antigens, could fulfill this task. Therefore, Mabs are used as vehicles to deliver cytotoxic drugs selectively to tumour cells\textsuperscript{8,9}. A drug-Mab conjugate would target the tumour cells by binding to the antigens on their surfaces. The conjugate is then internalized in active form\textsuperscript{10-12}. This type of immunoconjugate can be categorized as a...
“tumour-activated prodrug” (TAP)\(^1\). In this work, paclitaxel (Taxol\(^{®}\)), currently used as anticancer drug for the treatment of breast, ovarian and other cancers, is used for making drug immunoconjugate with a specific antibody to EGFR expressing cancer cells. The paclitaxel is derivatized at two different positions and linked to antibody. The purified paclitaxel immunoconjugates were evaluated. The in vitro cytotoxic evaluation of the drug immunoconjugates indicated the possibility of this approach for therapy.

Materials and Methods

Chemicals
Taxol (paclitaxel) was purchased from Dabur India Ltd. 1-(3-dimethyl amino propyl)-3-ethyl carbodiimide and t-butyl dimethyl silyl chloride were purchased from Sigma Aldrich (USA); succinic anhydride was purchased from Kemphasol, Mumbai, India; N-hydroxyl succinimide was purchased from Lancaster synthesis (England), MTT [3-(4,5-di-methylthiazol-2yl)-2,5-diphenyltetrazolium bromide] and RPMI 1640 were purchased from Himedia, Mumbai, India; HPLC grade acetonitrile and precoated TLC plates with silica gel F254 were purchased from Merck, Mumbai, India. All the other chemicals used were of analytical research grade.

Cell Lines and Monoclonal Antibodies
A-549 (Lung adenocarcinoma) was a gift from JSS College of Pharmacy, Ooty, Tamilnadu, India. HBL-100 (breast cancer) was a gift from Dabur Research Foundation, Ghaziabad, India. MCF-7 (breast cancer) was a gift from CCMB, Hyderabad, India. Murine monoclonal antibody (CIBCNSH3) was a kind gift from Dr A Meenakshi, Cancer Institute, Chennai, India.

Methods

Synthesis of derivatives of taxol and purification
Succinyl derivatives of taxol at 2′-OH and 7-OH groups were prepared as per the procedures of Deutsch et al and Magri & Kingston\(^{13,14}\). The reaction steps for derivatisation Schemes 1 and 2 are shown in Figs 1 & 2. In brief, the 2′-succinyl taxol (2′ST) was prepared in a single step with succinic anhydride in pyridine at room temperature. The 7-succinyl taxol (7ST) was prepared in two steps. In first step, 2′-OH group was protected by silylation. In second step, 7-OH group was succinylated. The progresses of the reactions were monitored by TLC technique. Both the products were purified by column chromatography with silica gel (230-400 mesh) using mobile phases benzene and ethyl acetate in ratios of 2:8 in case of 2′-succinyl taxol while 8:2 in case of 7-succinyl taxol. Further, the formed derivatives were checked by analytical HPLC (Shimadzu, Japan) method using C18 column (Phenomenex) with mobile phase consisting of acetonitrile and water (60:40) for taxol and 7-succinyl taxol. In case of 2′-succinyl taxol the mobile phase was acetonitrile and water (45:55). The structures of the derivatives were confirmed by comparing with the published \(^1\)H-NMR (Gemini 200 MHz) spectroscopic data\(^{14}\).

Preparation of taxol drug immunoconjugates (DICs) and their quantitation
The 2′-succinyl taxol and 7-succinyl taxol immunoconjugates were prepared with Mab CIBCNSH3 against EGF receptors on cancer cells using the procedure involving water soluble EDC method\(^{14,15}\). The corresponding reaction steps are given in the schemes 1 and 2. In brief, in this method, 2′-succinyl taxol/7-succinyl taxol was treated with EDC and N-hydroxy succinimide in dry DMF for about 1 h at 25°C. Then the solution of Mab CIBCNSH3 was added and the mixture was stirred for 16 h at 4°C. The unbound taxol derivatives were separated by either dialysis or centricron separators.

Quantitation of Drug immunoconjugate

Method 1
2′-succinyl taxol and antibody ratio in drug immunoconjugate was estimated by extracting the linked paclitaxel after hydrolysis of conjugate and comparing with the standard concentrations of pure paclitaxel. A known amount of taxol immunoconjugate was lysed using 0.1M acetate buffer (pH 4) for 48 h at room temperature. The taxol was extracted with chloroform, dried and reconstituted in methanol\(^{16}\). The quantity of paclitaxel was estimated using analytical HPLC method with following conditions. Column-Phenomenex (C-18), Wavelength-228 nm, Flow rate-1 mL/min, Pressure-87-93 psi, Mobile phase-Acetonitrile:Water-60:40, Retention time-7.465 min.

Method 2
7-succinyl taxol and antibody ratio in the taxol immunoconjugate was estimated by UV spectrophotometer using simultaneous Beer’s law equation.
after estimating standard concentrations of 7-succinyl taxol at $\lambda_{\text{max}}$ 276 nm and Mab at $\lambda_{\text{max}}$ 280 nm.

**In vitro Evaluation for Cytotoxic Activity of Drug Immunoconjugate using MTT Assay Method**

MTT assay method$^{17}$ was used to study cytotoxicity of drug immunoconjugates in comparison to taxol, 2'-succinyl taxol, 7-succinyl taxol and Mab on A-549 (lung adenocarcinoma), MCF-7 (breast carcinoma) and HBL-100 (breast carcinoma) expressing EGFR using media with less than 1% DMSO as control. The inhibitory concentration 50% (IC$_{50}$) values were calculated.

**Results and Discussion**

The RF values of taxol, 2'-silyl taxol and 7-succinyl taxol with mobile phase benzene and ethyl acetate in 8:2 ratios were found to be 0.09, 0.41 and 0.2, respectively. The RF values of taxol and 2'-succinyl taxol with mobile phase benzene and ethyl acetate in 2:8 ratios were found to be 0.75 and 0.2, respectively. In the HPLC profiles (not shown) of the taxol and 7-succinyl taxol and 2'-succinyl taxol retention times were found to be 7.0 min, 8.12 min and 3.29 min, respectively. The $^1$H NMR (200 MHz) spectra of 2'-succinyl taxol showed a strong peak at 2.6 ppm, 2'-silyl taxol showed a strong and characteristic peak.
at 0.02 ppm and no proton signal for 2'-OH group and for 7-succinyl taxol a strong peak at 2.6 and 2.25 ppm and proton signal at 4.78 ppm indicated the formation of 2'-succinyl taxol, 2'-silyl taxol and 7-succinyl taxol, respectively as already reported data\textsuperscript{14}.

The conjugation ratio of taxol immunoconjugate was found to be 0.45:1 for 7-succinyl taxol immunoconjugate and 0.38:1 for 2'-succinyl taxol immunoconjugate. The conjugation ratio was not improved after repeated trials. MTT assay method was used to study cytotoxicity of DIC on A-549 (lung adenocarcinoma), MCF-7 and HBL-100 (breast carcinoma) using media with <1% DMSO as control. The cytotoxic activities at different levels on three
different cancer cells are shown in Figs 3-5. Upon
derivatisation, cytotoxic activity of 2′-succinyl taxol
as compared to pure taxol was slightly less and it was
attributed to the blocking of 2′-OH group by
2′-succinylation (8) but 7-succinyl taxol showed more
cytotoxicity as compared to pure taxol indicating the
7-OH group was not biologically crucial even though
it was derivatized. The 7-succinyl taxol and
2′-succinyl taxol immunoconjugates showed more
cytotoxicity in comparison to the free Mab on the
cancer cells, as indicated by the results of MTT assay.
Probably, the antibody in drug immunoconjugates
carried the taxol derivatives to the cells, increasing the
internalization leading to the enhanced cytotoxic
effect.

The IC$_{50}$ determinations were conducted for drug
immunoconjugates on different cancer cell lines
(Figs 6-8) and the values are represented as bar
diagrams. The IC$_{50}$ values of Mab, CIBCNSH3
differed for different cell lines indicating that there is
differential expression of EGFR on these cell lines.
This altered preference was reflected on the efficacy
of DICs also. In general when compared, the amount
of 7-succinyl taxol immunoconjugate or 2′-succinyl
 taxol immunoconjugate required is nearly half of the
amount of pure Mab required to get the IC$_{50}$ on the
different cell lines. The IC$_{50}$ value of 7-succinyl taxol immunoconjugate, 70.4 nM was less than that of pure Mab, 161.7 nM on A-549 cells (Fig. 6). The 2'-succinyl taxol conjugate was tested on two different cancer cell lines MCF-7 and on HBL-100 cells. The 2'-succinyl taxol immunoconjugate was having IC$_{50}$ value of 120.3 nM as compared to the Mab having 236.5 nM (Fig. 8). In this case, the DIC required is almost 50% concentration to get the IC$_{50}$. On MCF-7 cells, the 2'-succinyl taxol conjugate was requiring 114.8 nM (Fig. 7) to get IC$_{50}$ as compared to that of Mab i.e., 267.3 nM. Here also, the activity of DIC is very much high when compared to Mab and required less than 50% concentration. Our results confirmed the efficacy of DICs over the Mab. However, the in vivo tests should be performed in tumour induced nude mouse models to know the real efficacy of taxol drug immunoconjugates.

Conclusion
The objective of preparing taxol drug immunoconjugates has been accomplished. As conjugation ratio was relatively low, additional efforts are required to increase the drug antibody conjugation ratio. In addition, different protocols may be tried to improve the drug to the antibody ratio in the immunoconjugate. The in vitro studies revealed that there is an increase in cytotoxic activity of the taxol drug immunoconjugates as compared to pure Mab.

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References