Original Article

Optimization of the Assembly Efficiency for Lidamycin Chromophore Bound to its Apoprotein: A Case Study using Orthogonal Array*

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Abstract

Objective Lidamycin (LDM) can be dissociated to an apoprotein (LDP) and an active enediyne chromophore (AE). The detached AE can reassemble with its LDP-containing fusion protein to endow the latter with potent antitumor activity. However, the reassembly of AE with LDP is affected by several factors. Our aim was to optimize the assembly efficiency of the AE with a LDP-containing fusion protein and investigate the influence of several factors on the assembly efficacy.

Methods A method based on RP-HPLC was developed to analyze the assembly rate, and an orthogonal experimental design $L_9(3^4)$ was used to investigate the effects of temperature, assembly time, pH and molecular ratio of LDP-containing fusion protein to AE on the assembly rate. Furthermore, the determined optimum conditions for the assembly rate of the LDP-containing fusion protein with AE were applied and evaluated.

Results A calibration curve based on the LDM micromolar concentration against the peak-area of AE by HPLC was obtained. The order in which individual factors in the orthogonal experiment affected the assembly rate were temperature>time>pH>molar ratio of AE to protein and all were statistically significant (P<0.01). The optimal assembly conditions were temperature at 10 °C, time of 12 h, pH 7.0, and the molar ratio of AE: protein of 5:1. The assembly rate of AE with a LDP-containing fusion protein was improved by 23% after condition optimization.

Conclusion The assembly rate of chromophore of lidamycin with its LDP-containing fusion protein was improved after condition optimization by orthogonal design, and the optimal conditions described herein should prove useful for the development of this type of LDP-containing fusion protein.

Key words: Lidamycin; Chromophore; HPLC; Orthogonal array; Assembly rate

Biomed Environ Sci, 2011; 24(6):602-607 doi:10.3967/0895-3988.2011.06.003 ISSN:0895-3988

www.besjournal.com(full text) CN:11-2816/Q

INTRODUCTION

Lidamycin (LDM, also named C-1027), a member of the enediyne antibiotic family, is 1 000-fold more potent than conventional cytotoxic drugs[1]. However, one inevitable issue of almost all potent cytotoxic agents is that their use is dose-limiting because of high systemic toxicity. Therefore, we seek to develop an appropriate vehicle to deliver LDM to tumor sites for therapeutic purposes. The development of an antibody-drug immunoconjugate has been proven to be effective against cancer as it works to enhance the therapeutic efficacy of the conjugated drug via antibody targeted delivery while reducing overall systemic toxicity[2].

*This research was supported by grants from "Significant new drug development" Science and Technology Major Projects of China (2009ZX09301-003; 2009ZX09401-005; 2010ZX09401-407).

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Received: January 13, 2011; Accepted: May 13, 2011

LDM is composed of an active enediyne chromophore (AE) and an apoprotein (LDP) in a 1:1 stoichiometry[1,3]. The AE portion possesses the potent antitumor activity and the LDP part mainly acts as the carrier of AE. These two moieties, connected to each other by non-covalent binding, can be dissociated and reconstituted under certain condition[4]. We created a series of fusion protein composed of an antibody fragment and the LDP by genetic engineering which could assemble with the detached AE to obtain a combination with potent antitumor activity[5-8]. This genetically engineered antibody-directed delivery approach can produce a more uniform and stable product compared to the chemically coupled conjugates of antibody with the intact LDM[9-10], which were a mixture of heterogeneous products like other antibody-drug immunoconjugates[11]. Nevertheless, a LDP-containing fusion protein introduces another issue—the assembly efficiency of the LDP-containing fusion protein with the chromophore AE. The assembly of the AE with the fusion protein is affected by several factors and needs further investigation.

We previously reported the optimal conditions for the reconstitution of LDP with AE which were obtained using a tumor cell clonogenic assay in a one-factor-at-a-time pattern. It was found that the temperature, assembly time and pH all influenced the assembly efficacy[4]. However, how exactly each factor influences the assembly efficacy is still unclear, and is complicated by possible interactions among these several factors and the time cost of the clonogenic assay used for condition optimization.

Orthogonal array design is a highly efficient method to investigate the relationship between the effects of different factors, and is often adopted for condition optimization in drug development[12-13]. In this study, a chromatographic method based on RP-HPLC was developed which was used to analyze the assembly rate of AE with the LDP-containing fusion protein. Then we used the assembly of a LDP-containing fusion protein, dFv-LDP (a tandem anti-gelatinase scFv fused to the LDP[7]) with the chromophore AE, as a case study to investigate the influence of several factors on the assembly efficiency by orthogonal array. We attempted to obtain the optimal assembly conditions of dFv-LDP with AE after the analysis of orthogonal experimental results and thereby laid a foundation for the efficient assembly of other LDP-containing fusion proteins with the chromophore AE.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile, TFA, and other reagents were analytical grade, and were obtained from local chemical companies. The preparation of dFv-LDP and AE were described as previously reported[7].

Calibration Curve

The RP-HPLC was carried out as described previously[7]. A series of concentrations of the intact LDM were analyzed by HPLC, and then a calibration curve was obtained based on the relationship of the concentration of LDM protein and the peak-area of the chromophore. The calibration curves were generated by linear regression using Microsoft Excel, where *x* was the concentration of lidamycin, and *y* was the integrated peak-area of AE. The calibration standards of these concentrations, namely 4.4, 8.8, 17.6, 35.2, and 70.4 μmol/L, were used to generate the calibration curve and assess the linearity. During quantification of the actual samples from assembly efficiency analysis, the samples with protein concentration >70.4 μmol/L would be appropriately diluted with solvent blank to fall within the calibration range.

Orthogonal Assay

As the temperature, assembly time, Ph, and the molar ratio of AE to dFv-LDP would likely influence the assembly rate[4], three levels for each factor, which were fixed in the probable working range, were selected. The $L_9(3^4)$ table of orthogonal design was used to test the assembly rate of dFv-LDP with AE under the assigned conditions (Table 1).

The procedures of orthogonal design assay used for optimization of the assembly efficacy of AE with LDP-containing fusion protein dFv-LDP were carried out as follows. The AE fraction was mixed with the dFv-LDP at different molar ratios (1:1, 3:1, or 5:1) in a brown conical flask and pH was adjusted with 1 mol/L sodium phosphate buffer to pH 6.0, 7.0, or 8.0 and then, the flask was placed on a rocking bed (60 rpm) at three different temperature (10, 20, or 30 °C) for different times (6, 12, or 18 h). The assembled product fraction containing dFv-LDP-AE prepared under different conditions was separated by

Sephadex G-25 gel-filtration chromatography by following the UV absorbance at 280 nm. Then the purified dFv-LDP-AE was analyzed by HPLC and the peak-area of AE was integrated. From this, the assembly rate can be calculated using the calibration curve as well as the corresponding molar concentration of dFv-LDP-AE, which was determined by the protein concentration divided by the molecular weight of dFv-LDP-AE. The protein concentration was determined using the BCA™ protein assay kit (Pierce, USA) and the molecular weight of dFv-LDP-AE was about 67 kDa.

ANOVA statistical analysis was applied to test significance. Results were considered to be statistically significant at $P<0.05$. Finally, the best assembly conditions were selected based on the significant factors and the assembly experiment was performed under the optimized conditions.

MTT Assays

Tumor cells were seeded at 2 000 cells/well in 96-well plates (Costar) for overnight cultivation, the different concentrations of dFv-LDP-AE (prepared under optimized and non-optimized conditions) were added and incubated for an additional 48 h. The effects on cell growth were examined by MTT assay which was described previously[5]. Results of the quantitative data from the MTT assay are presented as the mean±SD. The data were compared among groups using a two-tailed unpaired Student's *t*-test. A significant level threshold of $P<0.05$ was adapted.

RESULTS AND DISCUSSION

Calibration Curve

We have previously evaluated the characteristics of LDM using RP-HPLC and determined that the chromophore could be extracted from the LDM protein complex. As shown in Figure 1A, the apoprotein was eluted out before 4 min and peak 1 with a retention time around 7.8 min was the chromophore and peak 2 with a retention time around 8.5 min was the decomposed form of chromophore as determined by ESI-MS (unpublished data, for the chemical structures of chromophore refer to [14]). On the basis of the aforementioned observations, a calibration curve could be plotted between the peak-area of the chromophore (the summed peak-area of peak 1 and peak 2) and the LDM micromolar concentration (Figure 1B). Based on the calibration curve, the assembly rate of AE with other LDP-containing fusion proteins can be determined.

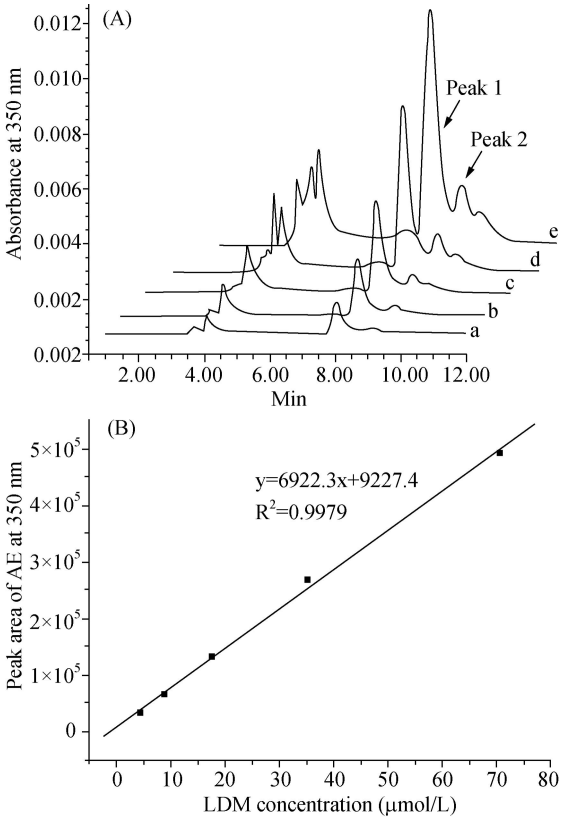

Figure 1. The calibration curves of peak-area of AE versus LDM concentration. (A) Chromatographic graph of LDM from RP-HPLC at 350 nm with different concentrations: (a) 4.4 μmol/L; (b) 8.8 μmol/L; (c) 17.6 μmol/L; (d) 35.2 μmol/L; (e) 70.4 μmol/L. Peak 1 with a retention time around 7.8 min was the AE and Peak 2 with a retention time around 8.5 min was the decomposed form of AE. (B) Plots of LDM micromolar concentrations against the peak-areas of chromophore (sum of Peak 1 and Peak 2) at 350 nm. The relationship could be expressed as $y=6922.3x+9227.4$ ($n=5$) with a correlation coefficient of 0.9979.

Orthogonal Array

It was found that the temperature, assembly time and pH influenced the assembly efficiency of LDP with AE, and the selected conditions for the reconstitution was pH 7.0 at 20 °C for 12 h[4]. By also

considering the molar ratio of the AE to dFv-LDP, an orthogonal design array $L_9(3^4)$ was selected to arrange these four factors within three levels. All of the factors are described in the following tables, and the detailed test procedure and results are listed in Table 1.

Among the tested four parameters after analysis of the experimental results (Table 2), temperature contributed the highest influence on the assembly rate (61.44%) and time and pH showed a moderate influence, 21.74% and 14.17% respectively. The molar ratio of AE to dFv-LDP fusion protein played a relatively less important role, with a 1.59% influence on the assembly rate. The order in which individual factors affected the assembly rate was temperature> time>pH>molar ratio of AE:dFv-LDP with all four factors being statistically significant (P<0.01).

From Table 1, the optimal conditions for assembly reaction were 10 ℃, 12 h, pH 7.0, and a molar ratio of AE:dFv-LDP at 5:1. However, the optimal combination was not presented within the nine groups of the orthogonal array. To confirm this combination, the assembly reaction of dFv-LDP with AE was performed under the above selected optimal combination and the final assembly rate was improved to 67%. In contrast, the assembly rate of dFv-LDP with AE was 44% under the pre-optimized conditions of 20 °C, 12 h, pH 7.0, and a molar ratio of 3:1 (AE:dFv-LDP). The assembly rate was increased by 23% after condition optimization using the orthogonal array. As shown in Figure 2 (b and c), the peak-area of chromophore AE was apparently increased under the optimized conditions given that protein concentration was constant.

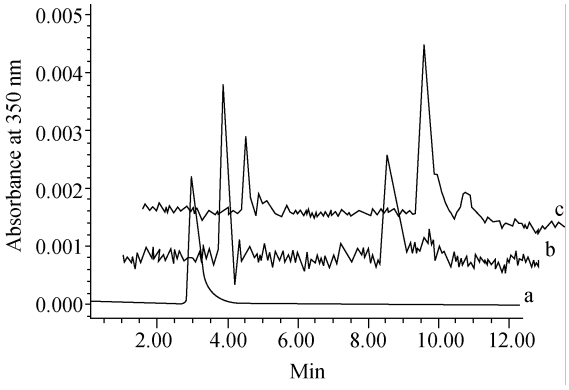

Figure 2. The HPLC chromatogram of the assembled product dFv-LDP-AE prepared under the conditions before or after optimization. a: the fusion protein dFv-LDP; b and c were chromatograms of the assembled product dFv-LDP-AE prepared under the pre-optimized and optimized conditions respectively.

Table 1. Results of $L_9(3^4)$ Orthogonal Design Array

Number[a]	Temperature	Time	Ratio[b]	pH[c]	Assembly Rate (%)[d]			Total (%)
1	1 (10 °C)	1 (6 h)	1 (1:1)	1 (6.0)	15.3	13.2	13.8	42.3
2	1 (10 °C)	2 (12 h)	2 (3:1)	2 (7.0)	41.7	48.3	45.9	135.9
3	1 (10 °C)	3 (18 h)	3 (5:1)	3 (8.0)	43.8	41.9	38.5	124.2
4	2 (20 °C)	1 (6 h)	2 (3:1)	3 (8.0)	23.8	22.7	26.1	72.6
5	2 (20 °C)	2 (12 h)	3 (5:1)	1 (6.0)	33.4	27.9	34.1	95.4
6	2 (20 °C)	3 (18 h)	1 (1:1)	2 (7.0)	42.8	37.3	41.1	121.2
7	3 (30 °C)	1 (6 h)	3 (5:1)	2 (7.0)	5.8	7.3	7.8	20.9
8	3 (30 °C)	2 (12 h)	1 (1:1)	3 (8.0)	11.9	9.3	15.5	36.7
9	3 (30 °C)	3 (18 h)	2 (3:1)	1 (6.0)	6.3	4.7	8.8	19.8
I	3.034	1.364	2.008	1.581				
II	2.892	2.684	2.287	2.784				
III	0.774	2.652	2.405	2.335				
R	2.260	1.320	0.397	1.203				

Note. [a]According to the orthogonal array design, 9 groups for assembly of dFv-LDP with AE were studied. The final reaction volume of each group was 1 mL containing 0.5 mg dFv-LDP fusion protein, each group was done in triplicate; [b]The ratio was the molar concentration of AE to dFv-LDP; [c]The pH of the reaction solvent was adjusted by 1 mol/L sodium phosphate buffer to pH 6.0, 7.0, and 8.0; [d]The assembly rate was deduced from the actual peak-area versus the theoretical peak-area which was obtained using the calibration curve and the protein molar concentration as described in Materials and Methods.

Table 2. Analysis of Variance (ANOVA) of Experimental Factors Influencing the Assembly Rate

	Sum of Squares	DOF	Variance	*F*-value	Significance	Percent (%)
Temperature	0.3561	2	0.1781	523.8	*P*<0.01	61.44
Time	0.1260	2	0.0630	185.3	*P*<0.01	21.74
Ratio	0.0092	2	0.0046	13.5	*P*<0.01	1.59
pH	0.0821	2	0.0411	120.9	*P*<0.01	14.17
Se2	0.0061	18	0.00034			1.05

Note. DOF: degrees of freedom; $F_{0.05}(2,18)=3.55$, $F_{0.01}(2,18)=6.01$; *P*: level of significance.

The Cytotoxic Assay In Vitro

The cytotoxic activity of assembly product dFv-LDP-AE prepared under the optimized and pre-optimized conditions *in vitro* was determined by the MTT assay. As shown in Table 3, the cytotoxic activity of dFv-LDP-AE was enhanced significantly (*P*<0.05) after condition optimization which indicated that the antitumor effects might be improved *in vivo*.

Table 3. The IC_{50} Values of dFv-LDP-AE Prepared under the Pre-optimized and Optimized Conditions

Groups	IC_{50} (mean±SD,×10^{-11}, mol/L)		
	HCT-116	HepG 2	Bel-7402
dFv-LDP-AE (pre-optimized)	3.44±0.50	1.03±0.10	6.61±0.23
dFv-LDP-AE (optimized)	1.22±0.27	7.62±0.08	4.42±0.53

CONCLUSION

A HPLC method was established to analyze the assembly rate of the chromophore AE with its apoprotein-containing fusion protein dFv-LDP. It was fast and convenient to determine the assembly rate of the dFv-LDP with AE using the established HPLC method, and the HPLC calibration curves exhibited good linearity in a defined range of standard concentrations and the correlation coefficient was nearly equal to 1.

The adopted orthogonal design $L_9(3^4)$ array in this study had facilitated a systematic mathematical approach to estimate the interactions of dFv-LDP and AE through nine groups of well-defined experimental sets. Among the four factors considered, temperature, time and pH had high impacts in determining the assembly rate in comparison with the molar ratio. The temperature showed the highest severity index compared with the other factors. The assembly rate of dFv-LDP with the AE under the optimized conditions was increased by 23% and the cytotoxic activity was enhanced as determined by the MTT assay.

In conclusion, this study investigated the influence of several factors on the assembly efficiency of dFv-LDP with AE by orthogonal array and then optimized them to improve the assembly efficiency. As the tumor-killing activity of the assembled product dFv-LDP-AE is correlated with the assembly rate, the *in vivo* administered dosage is dependent, in part, on the rate of AE loading. Therefore, the results demonstrated in this study will be useful for the analysis of assembly rate and the assembly procedure of chromophore AE with other apoprotein-containing fusion protein. This should help to lay a foundation for the development of this kind of antibody-lidamycin fusion protein in the future.

ABBREVIATIONS

LDM, lidamycin; LDP, apoprotein of lidamycin; AE, active enediyne chromophore; scFv, single chain variable fragment; dFv-LDP, a fusion protein consisting of a tandem scFv and the LDP; dFv-LDP-AE, the assembled product of dFv-LDP with AE.

ACKNOWLEDGMENTS

We thank Professor JIN Lian Fang, LIAN Run Mei, and ZHANG Zhong Yue for their assistance with HPLC analysis.

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