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Mini-Review

Immunochemical Analysis of the Antimalarial Drugs Artemisinin and Artesunate

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Abstract: We prepared a monoclonal antibody (mAb 1C1) showing specificity for artemisinin (AM) and artesunate (AS), and we developed an indirect competitive enzyme-linked immunosorbent assay (icELISA) using this novel mAb. Moreover, we prepared a recombinant antibody derived from mAb 1C1 in order to overcome insufficient mAb production by hybridoma culture. A recombinant antigen-binding fragment (Fab) was easily constructed using antibody manipulation technologies and was produced by microorganisms in high yield. We herein review immunochemical approaches for analysis of the antimalarial drugs AM and AS that were able to yield analysis results for multiple samples in a short period of time using simple and reliable protocols.

Keywords: artemisinin; artesunate; *Artemisia annua*; monoclonal antibody; recombinant Fab; ELISA

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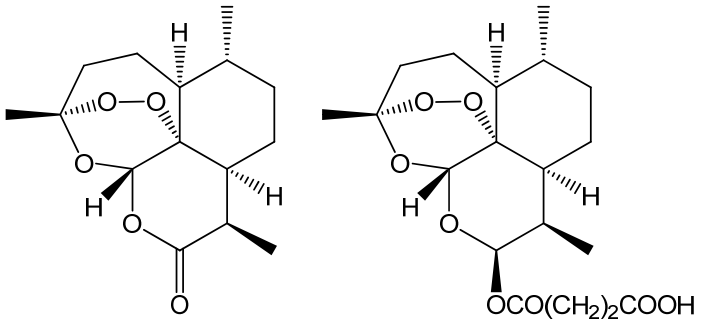
1. Introduction

Malaria is a devastating disease that represents a worldwide health threat, particularly in sub-Saharan Africa and South-East Asia. According to the 2010 World Malaria Report, the number of deaths due to malaria in 2009 was 781,000. Thus, international funding for malaria control has risen steeply in recent decades, reaching US $18 billion in 2010.

Artemisinin (AM), which is a sesquiterpene lactone, is a well-known antimalarial compound isolated from *Artemisia annua* [1–3]. Currently, AM and its related compounds (Figure 1), artesunate (AS) and artemether, are the most important drugs for the treatment of malaria and are used in artemisinin-based combination therapies (ACTs) recommended by the World Health Organization [4–8]. The number of patients receiving an ACT treatment course reached 158 million in 2009, and the cultivation of *A. annua* and production of artemisinin have expanded in line with the increase in global demand for ACTs.

Figure 1. Structures of artemisinin (AM) and artesunate (AS).



AM **AS**

ACT first-line treatments are 4–22 times more expensive than non-AM-based drugs. Because analogs of AM, as a class, show the highest efficacy toward the prevention of malaria epidemics, several synthetic organic chemistry and chemical biology groups have undertaken programs to develop a scalable synthetic approach for the preparation of AM; however, these programs have not yet devised a practicable AM supply system [9–11]. Demand for AM may therefore be better met by isolating compounds from natural sources, such as *A. annua*.

In addition to its antimalarial properties, AM is of interest for its anticancer effects *in vitro* and *in vivo* [12]. Recent studies have shed light on the mechanisms underlying the anticancer activities of AM, and synthetic approaches to preparing AM derivatives that display better efficacy and tolerability profiles have been conducted by several groups [13–20]. The expanded medicinal applications of AM-related compounds may increase the demand for AM in the future. The efficient production of AM from good quality *A. annua* and the appropriate use of AM-related compounds is one practical strategy for alleviating the economic burden to patients and society.

Some research groups have pursued development of immunochemical approaches by designing a convenient indirect competitive enzyme-linked immunosorbent assay (icELISA) using polyclonal antibodies or monoclonal antibodies for the analysis of AM and its related compounds [21–24]. Immunoassays are convenient and enable the rapid analysis of multiple samples [25–33]. We have

developed quality control methods for preparing AM from an *Artemisia* spp. selected for its ability to produce high yields of AM [34].

We recently devised and characterized two types of recombinant antibodies against AM and AS, and we evaluated their utility in an enzyme-linked immunosorbent assay (ELISA) for the detection of AM-related compounds produced in an *Artemisia* spp. culture [35]. We herein report the development of a sensitive and reliable immunochemical analysis using the mAb and recombinant antibodies for the measurement of AM and its derivatives.

2. Establishment of icELISA Using Anti-AM mAb

2.1. Production and Characterization of Anti-AM mAb

AM is poorly immunogenic because it is a low molecular weight compound, a so-called hapten. Generally, for the production of antibodies against haptens, a hapten-conjugate protein is used as an immunogen. Thus, we attempted to prepare an adequate immunogen for the production of an antibody against AM. AS, which is not only a structurally related compound of AM but is also one of the most important antimalarial drugs, was selected as an ideal hapten to prepare a conjugated protein, asAM has no functional groups. AS, which has a carboxylic group, could be easily conjugated with a carrier protein in the presence of EDC.

BALB/c mice hyperimmunized with AS-BSA yielded splenocytes producing an antibody specific to AM, and these were fused with SP2/0 myeloma cells using a routine procedure established in our laboratory. After testing the reactivity of each mAb with direct ELISA employing AS-HSA as a solid-phase antigen, hybridomas producing mAb reactive to AS-HSA were selected. The following icELISA was then performed in order to confirm the reactivity of each mAb against AM and eventually one hybridoma (1C1) secreting an mAb recognizing AM was obtained.

A purified mAb is required to confirm the reliability and reproducibility of the assay. After obtaining cloned hybridoma 1C1, a large-scale culture was implemented and an adequate amount of anti-AM mAb 1C1 was purified from the culture supernatant by Protein G affinity chromatography. The isotype of the anti-AM mAb 1C1 was then investigated using a purified mAb. Isotyping experiments classified the anti-AM mAb 1C1 having light chains into the IgG1 subclass antibody group.

2.2. Evaluation and Application of icELISA Using Anti-AM mAb

An icELISA using anti-AM mAb 1C1 was developed based on the general format that we developed previously [34]. The free mAb 1C1 after competition with AM is bound to polystyrene microtiter plates precoated with AM-HSA (1 μg/mL) in the ELISA. During incubation with a secondary antibody, mAb 1C1 was labeled with peroxidase. After addition of substrate solution (ABTS), absorbance values were measured at 405 nm and a standard curve was prepared using the known concentration of AM. The standard curve exhibited a sigmoidal decrease according to the increase in AM concentration. Under these conditions, the full measurement range of the assay for AM extends from 2 to 20 μg/mL.

An ELISA for AM should be applicable to phytochemical investigations involving crude plant extracts containing numerous secondary metabolites. Cross-reactivities of the mAbs used in ELISA are

thus an important factor to assess. The cross-reactivities of anti-AM mAb 1C1 were examined by competitive ELISA, and were calculated using the AM concentration yielding midrange values and the concentration of other compounds under investigation yielding midrange values by the method reported by Weiler and Zenk [36].

ELISA using mAb 1C1 was not specific for AM and showed cross-reactivities with AS and dihydroartemisinin, but did not react with other compounds. From these results, it appears that mAb 1C1 reacts with compounds whose basic skeleton is the same as that of AM and that it strongly recognizes the succinyl moiety of AS. The wide cross-reactivity of AM mAb 1C1 against AM-related compounds is a major advantage of the antibody reagent used in the ELISA, which is applied to both phytochemical investigations involving crude plant extracts and to therapeutic approaches with antimalarial drugs.

Table 1 shows the results of quantitative analysis of *A. annua* samples by ELISA. The results for AM content in Chinese wormwoods were similar to those reported previously [37,38]. However, other AM-related compounds which are recognized by the mAb 1C1 may affect these qualitative data. To confirm that these data reflect the content of AM in *A. annua*, we should compare them by the other analytical methods.

The benefits of this method are that it is easy to use and is capable of analyzing multiple samples at the same time without any sample pretreatment. The newly established ELISA using mAb-1C1may promote the breeding of high-AM-producing *A. annua* and prompt the appropriate therapeutic utility of antimalarial drugs.

Table 1. Content of artemisinin and its structurally related compounds in *Artemisia annua* L. samples determined by icELISA using anti-AM mAb 1C1.

Sample	Content (μg/mg dry wt.)
Chinese wormwood 1	3.21 ± 0.09
Chinese wormwood 2	1.02 ±0.23
Aerial part of regenerated *A. annua*	1.07 ± 0.05

The samples of *A. annua* are prepared and provided by Dr. Waraporn Putalun at Khon Kaen University, Thailand.

3. Production of Recombinant Fab against AM and AS

3.1. Cloning of cDNAs Encoding Fab and Bacterial Expression of Fab against AM and AS

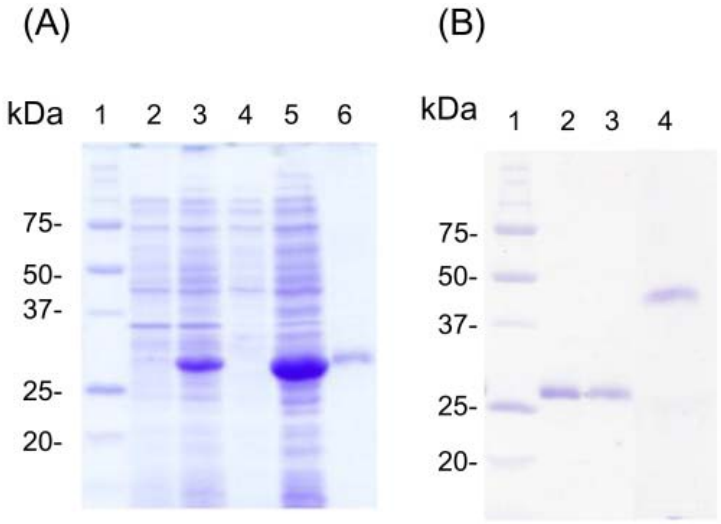
We prepared a monoclonal antibody (mAb 1C1) and developed an icELISA using this novel mAb. However, the yield of mAb 1C1 was insufficient due to the low production characteristics of the hybridoma, and this hindered the facile application of the novel antibody to various immunochemical experiments. To overcome the insufficient production of the mAb using hybridoma culture, we prepared a recombinant Fab against the antimalarial drugs AM and AS.

The cDNAs encoding the VH-CH1 and VL-CL fragments of the anti-AM mAb were amplified using two sets of specific primers containing an appropriate restriction site. Both cDNAs were successfully cloned in this way. The amino acid sequences were aligned to determine the genes encoding the VH and VL fragments by reference to the N and C termini of each fragment and the complementarity-determining region (CDR) using the Kabat and Chothia numbering scheme

(http://www.bioinf.org.uk/abs) [39,40]. Plausible genes encoding the VH and VL fragments of the anti-AM mAb 1C1 were identified. Both VH-CH1 and VL-CL genes consisted of 654 bp, and each fragment was fully consistent with the corresponding variable regions and constant regions of the IgG1 and a κ light chain of the anti-AM mAb, respectively, as determined by sequence analyses. The two cDNAs were inserted into a pET28a(+) vector for expression and characterization of the Fab against AM and AS.

Bacterial expression of VH1-CH1 and VL-CL using transformed *Escherichia coli* BL21 (DE3) was performed, and overexpressed proteins formed inclusion bodies. Each fragment with an N-terminal hexahistidine tag expressed in the insoluble fraction was resolved in an 8 M urea solution and was purified by metal chelator affinity chromatography. They were obtained in excellent yields, 19 mg per 1-L culture (VH1-CH1) and 18 mg per 1-L culture (VL-CL), respectively. A molecular weight of 27 kDa was determined by 12.5% SDS-PAGE (Figure 2B; Lanes 2 and 3), which was very similar to the theoretical values (27,305 Da for VH1-CH1 and 27,979 Da for VL-CL) predicted for each fragment with the tags.

Figure 2. SDS-PAGE analysis of recombinant antibodies against AM and AS. A) Analysis of the scFv. Lane 1, marker proteins; Lane 2, total protein before IPTG induction; Lane 3, total protein after IPTG induction; Lane 4, soluble fraction, Lane 5, insoluble fraction; Lane 6, purified scFv. B) Analysis of the Fab. Lane 1, marker proteins; Lane 2, purified VH-CH1; Lane 3, VL-CL; Lane 4, refolded Fab (non-reducing SDS-PAGE).



3.2. Refolding and Reactivity Tests for Fab against AM and AS

Because the purified recombinant antibodies were purified in their inactive forms, refolding was necessary in order to enable antigen recognition. We employed an efficient refolding method developed by Umetsu *et al.* [41] that is based on removal of the solubilizing agent by stepwise dialysis. Each recombinant antibody fragment that did not have a correct high-order structure was treated with β-ME in urea solution to cleave disulfide bonds, followed by dialysis against gradual tapering urea solutions. In the dialysis process, it is presumed that intramolecular hydrogen bonds are formed correctly and that hydrophobic interactions and van der Waals forces go on to facilitate their folding.

At the 2–0.125 M urea stages, L-arginine was supplemented as a solubilizing agent, and reduced and oxidized glutathione were added to facilitate the formation of intra- and intermolecular disulfide bonds. Figure 2B (Lane 4) shows the results of nonreducing SDS-PAGE analysis of the refolded Fab, in which only one band was observed. The results reveal that each supplement played a role in line with expectations and disulfide bonds formed during the refolding of the Fab, and regeneration of the binding capabilities were expected.

The sample was dialyzed to refold the Fab, and its reactivities were evaluated by indirect ELISA using an AS-OVA conjugate as the antigen. The validation study by indirect ELISA indicated that recombinant Fab recognized the AS-OVA conjugate, and optical density (OD) increased in a concentration-dependent manner. Therefore, we focused on the development and evaluation of an icELISA using the Fab for applications related to analysis of AM-related compounds.

3.3. Development of icELISA Using Recombinant Fab against AM and AS

In order to develop analytical methods for use during production of AM-related compounds, refolded Fab was used in an icELISA using AS-OVA as a solid-phase antigen. Figure 3 shows that the standard curves generated for AM were linear from 0.16 μg/mL to 40 μg/mL. The cross-reactivities (CRs) of Fab against other natural products were similar to those observed for the anti-AM mAb, which recognizes only AM-related compounds (Table 2). In case of analysis of AS, the linearity between concentrations of AS and the signal was observed from 8.0 ng/mL to 60 ng/mL. These results confirmed that the sequences of the variable fragments of the Fab yielded properties consistent with those of mAb 1C1.

Figure 3. Standard curves for the inhibition by AM in an indirect competitive ELISA using the recombinant Fab. Various concentrations of AM was incubated with the recombinant Fab (0.5 μg/mL) against AM in a 96-well immunoplate precoated with AS-OVA (1 μg/mL).

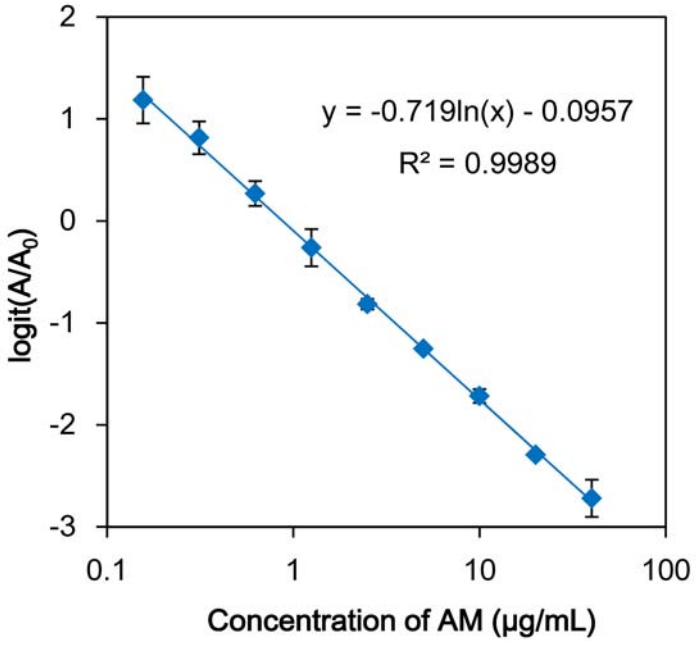


Table 2. Cross-reactivities of anti-AM Fab against various natural products.

Compound	IC_{50} (μg/mL)
Artemisinin	1.58
Artesunate	0.022
Saikosaponin a	>100
Ginsenoside Rb1	>100
Baicalin	>100
Quinine	>100
Berberine	>100
Arbutin	>100
Amygdalin	>100
Swertiamarin	>100
Digitonin	>100

We validated the icELISA using the Fab, as shown in Figure 3, for the quantitative identification of AM. The intra- and interassay variabilities were assessed for the icELISA by testing three samples of nine different AM concentrations performed together on the same day and on three consecutive days, respectively. The results showed that the maximum intraassay RSD was 4.7%, whereas the interassay RSD was 5.9%. The assay results were confirmed using the mAb, revealing that the developed icELISA was sufficiently accurate and reliable for quantitative analysis of AM.

After validating the icELISA as a quantitative method, the method was used for analysis of various *Artemisia* spp., which is the main source of AM. The leaves of five types of *Artemisia* spp. (*A. annua A. absinthium A. abrotanum A. iudovicianna A. capillaris, A. pontica*) were purchased from the market in Japan. They were dried and ground into powders, then extracted at room temperature with MeOH (0.5 mL) using an ultrasonic bath for 10 min. After centrifugation of the samples, the supernatant was transferred to a tube. This extraction step was repeated three times. After the extracted solutions had been concentrated *in vacuo*, the extract was dissolved in a small amount of MeOH and diluted appropriately to give 20% MeOH solutions for icELISA using the recombinant Fab against AM. As a result, AM content of *A. annua* was 5.40 μg/mg dwt., which was the highest value among the samples. The analytical data confirmed that *A. absinthium* and *A. abrotanum* contained AM. The experimental results were highly reproducible and agreed with previous reports, supporting the practical utility of the icELISA using the recombinant Fab against AM as a method for quantitatively analyzing AM [42].

4. Conclusions

Because AM and its structurally related compounds have poor UV chromophore or display fluorescence, previous quality control methods have involved derivatizing compounds in acidic or basic solutions for analysis using conventional TLC or HPLC techniques [43–47]. Recently, a liquid chromatographic tandem mass spectroscopy method for the quantification of AM was developed and was found to provide more sensitive quantitative analysis [48,49]. On the other hand, the benefits of this method also include its ease of use and its ability to analyze multiple samples at the same time without any sample pretreatment.

In addition, we developed expression systems that abundantly produce recombinant Fab against AM and AS, and we applied it to the icELISA in the first such approach to quantitative analysis of AM-related compounds. Given the poor production of anti-AM mAb by the hybridoma 1C1, the improved Fab production was significant for the development of a cost-effective immunoassay.

Our results suggest that the results encourage further evaluation of the technology as a potential method to assist with the development of AM plant breeding programs, quality checks in AM production operations, and techniques for therapeutic drug monitoring. The present immunoassay using antibodies possessing novel characteristics can potentially advance the clinical development of the anti-malarial drugs AM and AS.

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