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SCREENING INDOLOCARBAZOLE PRODUCING MICROORGANISMS BY MOLECULAR PROBES COMBINED WITH HPLC METHOD

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ABSTRACT

Indolocarbazole alkaloid constitutes a group of natural products which manifested powerful biological activity, especially anticancer. However, it was laborious to find ICZs-yield organism when anticancer activity was adopted as selection target. The specificity of 6 primer pairs was detected and the results revealed that primer rebBN1/rebBC1 possessed preferable specificity because indolocarbazole-producers DNA was exclusively amplified and no target fragment was amplified from any of indolocarbazole nonproducers. Therefore, primer rebBN1/rebBC1 could specifically identify indolocarbazole-producers from microorganism strains.

Key words: indolocarbazole, rebBN1/rebBC1, ICZs-producing microorganism screening, HPLC, probe

[I] INTRODUCTION

The indolocarbazole family is an important class of natural products isolated from bacteria, actinomycetes, cyanobacteria, fungi, slime moulds and marine invertebrates [1]. Since the discovery of staurosporine as the first ICZs in 1977, a variety of ICZs (more than 120) have been found and attracted great attention of chemists, biologists, physicians and pharmaceutical for their biological activities and the prospective number of derivatives from the basic backbone alone. Indolocarbazole compounds (ICZs) are structurally characterized by possessing an indolo[2,3-a]pyrrolo[3,4-c]carbazole core derived from two units of tryptophan, with sugars attached derived from glucose and methionine. According to their structural features and mechanisms of actions ICZs are divided into two major classes [2]. The first class was characterized by a sugar moiety linked to both indole nitrogen atoms of the indolocarbozole core to form a “closed” indol-carbazole and showed inhibition to protein kinase C, such as K-252a and staurosporine (STA). The

second class contains a sugar moiety attached only to one of the indole nitrogen atoms of the aglycone by a β-glycosidic linage to form an "open" bisindolymaleimide and exhibit inhibition to DNA topoisomerase I, such as rebeccamycin (REB) and AT2433. Despite the differences, it is assumed that the presence of the sugars in both classes appears to be essential for the biological activity of ICZ compounds [3].

ICZs display a wide range of biological activities including antibacterial, antifungal, antiviral, hypotensive , neuroprotective activities. However, the most attracting point is their therapeutic potential as anti-cancer drugs. Recently their strong effects to various harmful insects were discovered by our team (related article under review). Although several ICZs derivatives have already entered clinical trials for the treatment of cancer or other diseases, no analog has yet been launched onto the market and great efforts have been made to find or generate ICZs derivatives with improved properties for drugs. Despite the abundant diversity of ICZs-origin organisms, microbes were the main origin of most known ICZs. During the search for novel ICZs-producing organisms and the isolation of ICZs, blindness and repetitiveness have been the formidable obstacles with great loss of labor and time. In order to solve the problem, the feasibility of using molecular probes combined with HPLC to detect ICZs-producing microorganisms was investigated in this article.

[II] MATERIALS AND METHODS

2.1. Strains in this research

Totally 56 strains with potential pharmaceutical activity, including bacteria 10, actinomycetes 36, and fungi 10 deposited in China Pharmaceutical Marine Microbiological Databank (which was established by our team) were selected as test microbes. Among them, Streptomyces nitrosporus CQT14-24 (T14-24) was staurosporine-producing strain which was under intensive investigation in our lab. This strain was isolated from sediment of Chukchi Sea (the North Pole) and were deposited both in China Pharmaceutical Marine Microbiological Databank (No.HTTMS-F04008) and China General Microbiological Culture Collection Center (No.CGMCC 4607), and recorded at GenBank with the accession number FJ821473. Streptomyces nitrosporus CQT5-L25-1 (5-L25-1) was a mutant of T14-24 with increased yield of staurosporine and increased kinds of ICZs homolog. T14-24 and 5-L25-1 were used as positive control.

2.2. Culture conditions and DNA isolation

For ICZs extraction, bacteria, actinomycete and fungi strains were shaking cultured by using MYPD medium, Gauze No.1 medium, MPDA medium at 25℃ for 7 days, 12 days and 10 days respectively. For DNA extraction, strains were cultured by using above mentioned medium for 2 days. Bacteria and actinomycete DNA were isolated by standard CTAB method while fungi DNA was isolated through glass beads method [4].

2.3. Molecular probes adopted in this article

Different conservative sequences of ICZs gene cluster were sought among known literatures and selected primers were listed as Table 1. Primers rebBN1 / rebBC1 were initially designed to amplify rebD of rebeccamycin gene cluster from Streptomyces sp. TP-A0274 [5]. Primers CS035 / CS036 were designed to carry out in situ colony hybridization of a constructed cosmid library of S. longisporoflavus DSM10189 which was highly similar to rebD in L. aerocolonigenes [6]. Primers StaCN / StaCH and StaPN /StaPH were designed to amplify staC and staP in LecheValieria aerocolonigenes [7]. Primers RebD1 / RebD2 were designed to amplify rebO in L. aerocolonigenes ATCC 39243 [8]. Primers NGT1 / NGT2 were designed to amplify ngt (now named rebG) in L. aerocolonigenes ATCC 39243 [9].

Table 1 primers used in this study

Primer Name	Primer Sequence	Reference
rebBN1 rebBC1	**5'-GAAGAATTCGTSATGCTSCAGTACCTSTA-3'** **5'-CGAAAGCTTSAGGAASAGGTGGTGCTCSCC-3'**	[5]Onaka *et al*, 2002
CS035-StaD CS036-StaD	**5'-ATATAAGCTTGATGGCCCAGCACTTCGG-3'** **5'-TATCTAGACGGCGGGCGGAAGCGGTC-3'**	[6]Salas *et al*, 2005
RebD1 RebD2	**5'-GGAGAGCATATGAGCGTCTTCGACCTG-3'** **5'-GTCAAGCTTTCGCGGTCCTTCCGTTGC-3'**	[8]Howard-Jones *et al*, 2005
StaPN StaPH	**5'-GGAGAGCATATGCCATCCGCGACGCTGC-3'** **5'-GTCAAGCTTGGGGTGGCTGGCCGAGGG-3'**	[7]Howard-Jones *et al*, 2006
StaCN StaCH	**5'-GGAGAGCATATGACGCATTCCGGTGAGCGGACC-3'** **5'-GTCAAGCTTGCCCCGCGGCTCACGGGGCGCGGC-3'**	[7]Howard-Jones et al, 2006
NGT1 NGT2	**5'-TCGGAATTCATGGGGGCACGAGTGCTG-3'** **5'-AGGAAGCTTGAACGGGCCGACGAACCT-3'**	[9]Onaka et al, 2003

2.4. PCR Amplification

PCR amplifications were performed in 50μl containing 1μl templete, 1μl of each primer (10μmol/L), 1μl deoxynucleotide triphosphates (2.5mmol/L), 5μl 10× PCR buffer, 1.5μL $MgCl_2$ (1.5mmol/L), 1.5μL DMSO, 1.5μL bovine serum albumin (BSA) (0.1%), 0.25μL TaqDNA polymerase (2U). The cycling parameters were: initial denaturation for 5 min at 95℃, followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, and extension at 72℃ for 2 min, a terminal extension step of 72℃ for 15 min.

2.5. Isolation of ICZs

After fermentation, the culture broth of different strains was filtered through cheesecloth to separate it into supernatant and mycelia, the former was extracted with equal volume EtOAc for two times while the later was extracted with EtoAc (200ml) by supersonic method, both of the EtOAc solutions were concentrated under reduced pressure to afford an MeOH solutions (5ml), the concentration of indolecarbazole alkaloids in MeOH solutions was determined by HPLC method. The Analytical HPLC system was composed of Waters Acquity UPLC and BEH c18 column (1.7μm, 2.1×50mm), chromatographic condition: (0-9min, 5%-100%MeOH, 9-11min, 100%min, 0.5ml/min).

[III] RESULTS

3.1. ICZs producing microorganisms

Among 54 test strains, 4 strains (all actinomycetes) including Streptomyces sp. T9-33, Streptomyces nitrosporeus T13-12, Streptomyces sp. YH3-2, Streptomyces sp. YT1-28 were detected to yield ICZs compounds while other strain showed no production of ICZs. Therefore, there are totally 6 strains (T14-24 and 5-L25-1 as positive control) which produce ICZ compounds. The HPLC spectrum of the 6 strains was similar with difference of peak area. Among them, strain 5-L25-1 yields the maximum amount of ICZ (peak area 17089081) while strain YH 3-2 yields the minimum of ICZ (peak area 1311480) (Fig 1). The ultraviolet absorption spectrum of the 5 strain except 5-L25-1 was similar with the main component of staurosporine. As for 5-L25-1, the ultraviolet absorption spectrum showed two main components staurosporine and K252c (Fig 2).

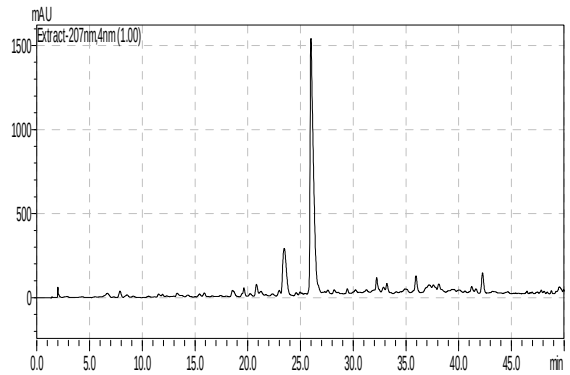
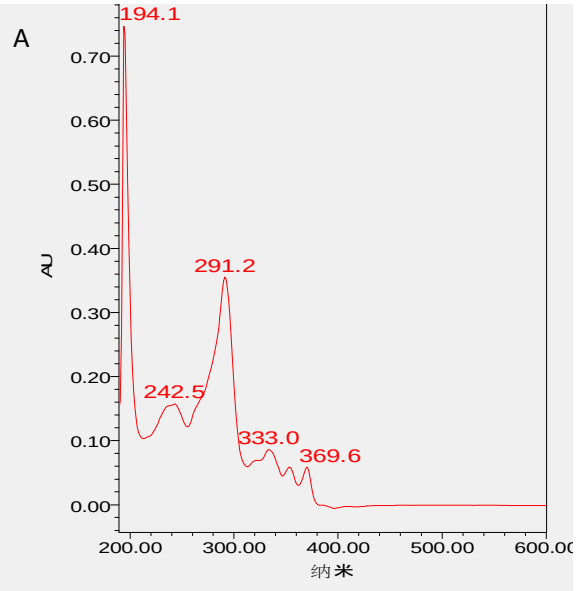
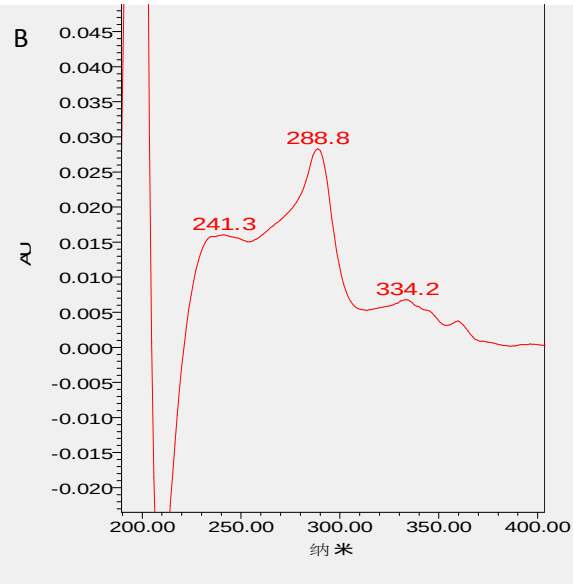


Fig 1 HPLC chromatograph a of ICZs producing microorganisms 5-L25-1



A: staurosporine



B: K252c

Fig 2 UV spectrum of ICZs producing microorganisms 5-L25-1

3.2. The specificity of different primers

Different ORFs fragments were amplified using specific primers and the results showed that for almost primers except StaPH/StaPN visible fragments were amplified only when chromosomal DNAs from indolocarbazole producers were used as templates. However, there was no regularity and specific indicator band(s) among the fragments bands except primers rebBN1/rebBC1 (Fig.3, A-E lane 1-6).

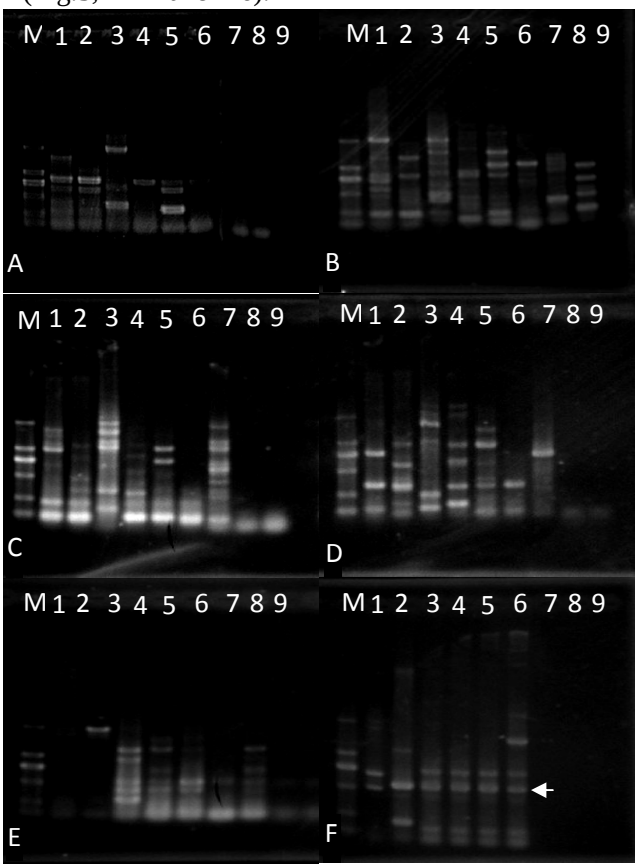


Fig 3 Sensitivity of different primers for detection of ICZs producing microorganisms

A: primers of RebD1/RebD2; B: primers of StaPH/StaPN;

C: primers of StaCN/StaCH; D: primers of CS035/CS036;

E: primers of NGT1/NGT2; F: primers of rebBN1/rebBC1

Lane M: DNA Marker 3, Lane 1-9: different strains : T14-24, 5-L25-1, 3-2, T13-12, T1-28, T9-33, Fc02, T9-37, G19-24 respectively

3.3. The specificity of primers rebBN1/rebBC1

The primers rebBN1/rebBC1 were synthesized using the highly conserved regions corresponding to amino acids from 611 to 618 and from 780 to 786 in RebD amino acid sequence and were supposed to produce fragment of 0.5-0.7 kb. As shown in Fig. 2F, a 0.5kb and/or 0.75 kb

fragments were amplified only when chromosomal DNAs from indolocarbazole producers were used as templates (Fig. 3F, lane 1-6). On the other hand, any DNA fragment with the size of neither 0.5kb nor 0.75kb was amplified with chromosomal DNAs from indolocarbazole nonproducers. The results demonstrated that this primers pairs possessed preferable specificity because indolocarbazole-producers DNA was exclusively amplified. Since no target fragment was amplified from any of indolocarbazole nonproducers, this primer pair could specifically identify indolocarbazole-producers from strains.

[IV] DISCUSSION

ICZs have attracted the attention of many researchers from different disciplines for their biological activities especially anticancer property. This property may be due to different mechanisms of action, including inhibition of protein kinases, DNA intercalation, and inhibition of DNA topoisomerases. Protein kinases and DNA topoisomerases are important in cell proliferation so specific inhibitors are promising candidates for antitumor or antifungi drugs and pesticide to kill harmful insects. Despite the high inhibition activities, certain known ICZs also exhibited high toxicity with low specificity which limited their direct clinical application. Therefore, great efforts have been made to search or generate novel ICZs derivatives with higher inhibitory specificity. Although organic chemistry methods are routinely used to synthesize and modify natural products, harvesting the product (or a modifiable precursor) from the natural source is often the only cost-effective way of production. Despite the abundant diversity of ICZs-origin organisms, most ICZs are isolated from actinomycetes: rebeccamycin is from *Lechevalieria aerocolonigenes* ATCC 39243 [10], staurosporine is from *L. aerocolonigenes* subsp. *staurosporeus* and *Streptomyces* sp. Strain TP-A0274 [11], K252a is from *Nocardiopsis* sp. Strain K252 [12]. Herein, actinomycetes were considered as the main test strains in this research along with certain bacteria and fungi strains and our results also verified this phenomenon. Among 54 test strains, 4 ICZs-yielding strains were all actinomycetes while no bacteria and fungus strains was detected to produce ICZs. Although the searching scope has been narrowed mainly to actinomycetes during the search for natural novel ICZs, it was still laborious to find ICZs-yield organism when anticancer activity was adopted as selection target. Therefore, in order to reduce the blindness, specific molecular probes might be feasible in effectively narrowing screening strains scope considering the fact that ICZs were biosynthsized similarly pathways and encoded by corresponding biosynthesis gene cluster.

Up to now, although the basic biosynthetic pathway for the indolocarbazole aglycone was revealed in the analysis of rebeccamycin and staurosporine biosynthesis, modified pathways for the individual indolocarbazole compounds remain unidentified. Studies on the biosynthesis of ICZs have shown that the indolocarbazole core is formed by decarboxylative fusion of two tryptophan-derived units, whereas the sugar moiety is derived from glucose [13,14]. Biosynthetic studies through isotope-labeled precursors indicated that the rebeccamycin and staurosporine indolocarbazole cores are derived from L-tryptophan (L-Trp) via a series of oxidative transformations. The initial pathways to the two aglycones follow very similar routes, differing only by the oxidation state at the C-7 position of aglycone and the pattern of connectivity between the deoxysugar and aglycone. Subsequent *N*-glycosylation and tailoring modifications follow divergent pathways toward rebeccamycin and staurosporine [7]. Biosynthesis of ICZs can be divided into five stages catalyzed by different sets of enzymes, which are (*i*) tryptophan modification

(halogenation) by RebH; (*ii*) dimerization by RebO/RebD or StaO/StaD; (*iii*) decarboxylative ring closure by RebC/RebP or StaC/StaP; (*iv*) glycosylation by RebG or StaG; and (*v*) sugar modification by RebM or several enzymes for staurosporine before and/or after glycosylation [10-13]refs. 10–13 and this article. Stages *ii* and *iii* constitute the central reactions in the pathway, whereas the other stages can be considered as accessories for ‘‘tailoring’’ the alkaloid skeleton [15]. In this sense, RebO/RebD (or StaO/StaD) and RebC/RebP (or StaC/StaP) play crucial role in the formation of the ICZs molecule backbone. On the other hand, some researchers consider glycosyl in ICZs is essential for its biological activities [15]. Therefore, we focus our intention also on genes encoded these two enzymes.

In recent years an increasing amount of information has become available on the molecular genetics of indolocarbazole biosynthesis. Until now, four kinds of ICZs biosynthetic gene clusters including rebeccamycin, staurosporine, AT2433, and K252a were cloned. The genetic organization of these four indolocarbazole gene clusters is quite similar and all of them contain genes required for the biosynthesis of the aglycone, biosynthesis and transfer of the deoxysugar and regulation. The rebeccamycin biosynthesis gene cluster was taking as example. On the basis of sequence analysis and database searches about rebeccamycin gene cluster from *Lechevalieria aerovolonigenes* ATCC 39243, Sanchez *et al* proposed that there are 11 open reading frames (ORFs) including four for ICZ-core biosynthesis (*rebO*, *rebD*, *rebC* and *rebP*), two for halogenation (*rebH* and *rebF*), glycosylation (*rebG*, the name was changed from *ngt*), and sugar methylation (*rebM*), as well as a regulatory gene (*rebR*) and two resistance and secretion genes (*rebU* and *rebT*) [16]. There were approximately counterparts in other three gene clusters. Considering the conservation in related gene clusters, gene fragment of different ORFs were adopted as molecular probe to detect ICZs producing microorganisms among bacteria and fungi strains which deposited in our lab. To be specific, primers rebBN1/rebBC1 and CS035/CS036 were designated to amplify *rebD/StaD* gene with about 0.3kb/0.5kb fragment. In the biosynthesis of indolocarbazoles, chromopyrrolic acid or 11,11'-dichlorochromopyrrolic acid is presumed to be an intermediate which might be directly biosynthesized by *rebD* which encodes a putative chromopyrrolic acid synthase [5,6]. Primers RebD1/RebD2 were designed to amplify *rebO* fragment which was predicted to be a flavoprotein encode gene [8]. Primers StaPN/StaPH and StaCN/StaCH were designed to amplify staP and staC, respectively [7]. RebP and RebC are responsible for the oxidative decarboxylation and ring fusion reactions that create the six-ring indolopyrrolocarbazole rebeccamycin aglycone [15]. The RebG (or StaG, once named Ngt) protein converted an indolocarbazole to its N-glucoside indicating that *RebG* (or *StaG*, once named *ngt*) is responsible for N-glycosylation in rebeccamycin biosynthesis [9]. An internal fragment of *ngt* was usually adopted to clone rebeccamycin biosynthetic genes as a probe. Based on these informations, 6 primer pairs were selected to amplify different ORF fragment for detection of ICZs-yield microorganisms. The results revealed that there were varied conservation degree among different ORFs, to be specific, *staD* (or *rebD*) possesses the maximum of conservation which may be owe to chromopyrrolic acid synthase is the key enzyme in the indolocarbazole aglycone synthesis and thus its existence is expected to be a useful indicator of indolocarbazole-producing strains. However, the conservation degree of different fragments in this ORF varied according to our results. Primer pair CS035/CS036 and rebBN1/rebBC1 were both designed to amplify

DNA fragment in *StaD* (or *rebD*), with designated region of 1-323 and 611-1110 respectively. The results revealed that fragment amplified by primer pair rebBN1/rebBC1 showed preferable conservation among ICZs-yielding microorganisms. What's more, because chromopyrrolic acid (or 11,11'-dichlorochromopyrrolic acid) might be the common intermediate of different kinds of ICZs, the primer pair could theoretically detect microorganism producing each kind ICZs.

On the other hand, although glycosylation is very important because biological activity of ICZs requires the presence of the sugar, the primer pair to amplify gene encode for glycosyltransferase *RebG* (or *StaG*, once named *ngt*) showed low specificity to detect ICZs-yielding microorganisms. This might because of the major structural difference between two kinds of ICZs: either through a single nitrogen (in the case of rebeccamycin) or through two nitrogens and this property might lead to the flexibility of glycosyltransferase which makes *RebG* (or *StaG*) improper probe region.

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