Genetic diversity of Merozoite Surface Protein-1 gene block 2 allelic types in *Plasmodium falciparum* isolates from Malaysia and Thailand

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Abstract. Malaria is the most common vector-borne parasitic disease in Malaysia and Thailand, especially in Malayan Borneo and along the Thailand border areas, but little is known about the genetic diversity of the parasite. Present study aims to investigate the genetic diversity of *Plasmodium falciparum* isolates in these two countries and eventually contributes to more effective malaria control strategies, particularly in vaccine and antimalarial treatment. One hundred and seventy three *P. falciparum* isolates were collected from Malaysia (n = 67) and Thailand (n = 106) and genotyped using nested PCR targeting the polymorphic region of *MSP-1*, block 2. Sequence analysis was conducted to investigate the allele diversity of the isolates. Three allelic families were identified in Malaysian and Thailand *P. falciparum* isolates, MAD20, K1 and RO33. Sequence analysis revealed that there were 5 different MAD20, 1 K1 and 2 different RO33 for Malaysian isolates. Thailand isolates exhibited greater polymorphism because there were 13 different MAD20, 6 different K1 and 2 different RO33 identified in this study. Multiclonal infections were observed for the isolates in both countries, however, low multiplicity of infection (MOI) was observed for Malaysian (1.1) and Thailand (1.2) isolates. Phylogenetic analysis showed that *P. falciparum* isolates of Malaysia and Thailand were clustered in the same group for all the allelic families. Population structure of *P. falciparum* isolates in Malaysia and Thailand exhibit extensive genetic polymorphism but showed high similarities as well as comparable MOI.

INTRODUCTION

Malaria, a tropical disease caused by infection with single-celled parasites of the genus *Plasmodium*, is one of the most deadly parasitic diseases in the world. According to World Malaria Report 2017, the disease had led to approximately 445,000 deaths and an estimated 285,000 children died globally before their fifth birthday due to this deadly disease in 2016 (WHO, 2017). In Malaysia, it is estimated that 4% of total Malaysians are at risk of malaria and 68% of total malaria cases occurred in Malaysian Borneo, in the states of Sabah and Sarawak while only one-third of cases were found in Peninsular Malaysia (Ministry of Health Malaysia, 2016). According to Vector-Borne Disease Control Sector, Ministry of Health Malaysia, over 90% of the indigenous human malaria cases were reported in Sabah (East Malaysia Borneo) (Vector-Borne Disease Control, 2014). As for Thailand, it was reported that around 33 million people are at risk of malaria (Bureau of Vector-Borne Disease, 2016) and most of the malaria cases were reported from and confined to provinces bordering neighbouring countries such as Thai-Myanmar borders,
ThaiCambodian border, ThaiMalaysian border and ThaiLao PDR border (Bureau of Vector-Borne Disease, 2010). Infection caused by *Plasmodium falciparum* is severe and may be life-threatening in the absence of prompt recognition of the disease and its complications, resulting in a majority of deaths (99%) (WHO, 2017). The morbidity and mortality rates due to *P. falciparum* have been declining gradually in recent years in Malaysia (William et al., 2013) and Thailand (Bureau of Vector-Borne Disease, 2010), but this disease continues as public health problem in the less developed areas of Malaysia and multidrug resistant *P. falciparum* remains one of the major health problems in Thailand.

Given that Malaysia and Thailand are neighbouring countries, extensive labour migration between these two countries may introduce a *P. falciparum* population with different alleles to the country, resulting in genetic recombination among the isolates, hence it is essential to know the genetic variability of the species strain circulating in these two countries in order to understand the dynamics of the disease transmission, genotypes of the circulating parasites. These data will eventually contribute to greater effective malaria control and prevention strategies, particular in vaccine and anti-malarial treatment. Besides, understanding the genetic structure of malaria parasite allows the prediction on how fast a phenotype of interest, such as novel antigenic variants or drug resistance, originate and spread in the populations (Zhong et al., 2007). Therefore, it is important to investigate the genetic makeup of *P. falciparum* in Malaysia and along the southeastern coast of Thailand in order to provide fundamental information on the genotypes circulating in these two countries.

The genetic diversity of *P. falciparum* is most studied by genotyping the surface proteins such as polymorphic region block 2 of merozoite surface protein-1 (*MSP-1*) (Atroosh et al., 2011), block 3 of the merozoite surface protein-2 (*MSP-2*) (Takala et al., 2006; Schoepflin et al., 2009), circumsporozoite protein (*CSP*) (Escalante et al., 2002), apical membrane antigen-1 (*AMA-1*) (Oliveira et al., 1996) and the RII repeat region of the glutamate rich protein (*GLURP*) (Mwingira et al., 2011). In this study, *MSP-1* was chosen to investigate the genetic diversity of falciparum isolates as it has been extensively used to study the genetic diversity of worldwide isolates, level of malaria transmission, multiplicity of infection and as a discriminatory tool to distinguish new from recrudescent infections. Merozoite surface protein-1 (*MSP-1*) of *Plasmodium* sp. is a large polypeptide (approximately 200 kDa) and the most abundant surface protein on the blood stage of *P. falciparum* and it plays an important role in erythrocyte invasion (Holder et al., 1992). Block 2, a region near the N-terminal of the *MSP-1* gene, is the most polymorphic part of the antigen and appears to be under the strongest diversifying selection within natural populations (Holder et al., 1994). At present, three different allelic types of block 2 have been identified including MAD20, K1 and RO33 (Kaur et al., 2017). Intragenic recombinations have been reported among these alleles, resulting in polymorphism among different isolates around the world (Conway & McBride 1991).

Currently, there have been only two studies carried out to investigate the genetic diversity of *P. falciparum* targeting the *MSP-1* gene in Malaysia, however, sequence analysis was not conducted in both studies and the samples were collected from only three locations i.e. Pahang (Atroosh et al., 2011), Kalabakan and Kota Marudu (Sabah Borneo) (Mohd Abd Razak et al., 2016). As for Thailand, two studies were carried out to investigate the genetic diversity of *P. falciparum* targeting the *MSP-1* gene block 2, however, sequence analysis was not conducted in both studies and the samples were collected from Thai-Cambodian (Snounou et al., 1999) and Thai-Myanmar border (Congpuong et al., 2014), with no information available for *P. falciparum* in southern Thailand. Therefore, the present study was conducted to examine the genetic makeup of *P. falciparum* in Malaysia and along the southeastern coast of Thailand.
targeting *MSP-1* block 2 as well as isolates from other countries. This information would provide a better understanding of genetic diversity of *P. falciparum* isolates in these two neighbouring countries. In this study, we have examined the allelic diversity of *P. falciparum* isolates in Malaysia and Thailand. Allele-specific PCR typing of this region showed that three types of allele families MAD20, K1 and RO33 were detected in both Malaysian and Thailand isolates.

### MATERIALS AND METHODS

#### Ethics Statement

This study was approved by the Ethics Committee of the University of Malaya Medical Centre, Malaysia (MEC Ref. No. 709.20).

#### Sample collection

In this study, a total of 173 *P. falciparum*-infected archived blood samples were collected from blood film for malaria parasites (BFMP)-positive patients who attended the hospital or malaria clinics in Malaysia and Thailand. The archived blood samples were collected over a period of seven years, from 2008 to 2014. Of the total samples, 67 samples were obtained from Malaysia while 106 samples were obtained from Thailand. Malaysia samples were collected from Hospital Kuala Kubu Bharu (Selangor), Telupid Health Clinic (Sabah) and its neighbouring districts (i.e. Kinabatangan, Tongod, Beluran and Sandakan) (Figure 1). Thailand samples were collected from malaria clinics located at southern Thailand (i.e., Ranong, Chumphon, Surat Thani and Yala) (Figure 2). Blood samples collected in this study were the leftover samples after routine diagnosis in respective hospital and clinics.

#### Conventional microscopy examination

Giemsa-stained thick and thin blood films and microscopy examination were performed by microscopist at respective hospital and clinic. Blood films of malaria suspected patients were prepared by finger prick blood samples prior to anti-malarial treatments.

#### Extraction of genomic DNA

Genomic DNA was extracted from blood sample of BFMP-positive patients using QiAamp® DNA Blood Mini Kit (Hilden, Germany), following the manufacturer’s recommendations. Extraction of genomic DNA and molecular analysis were conducted in University of Malaysia Sabah, University of Malaya (Malaysia) and Prince of Songkhla University (Thailand).

#### Detection of *Plasmodium* species using PlasmoNex™

All extracted DNA samples were confirmed for *P. falciparum* infection by using PlasmoNex™ (Reszon Diagnostics, Malaysia) targeting the 18S ssu rRNA gene (Chew *et al*., 2012). Blood samples infected by *Plasmodium* species other than *P. falciparum*, or by mixed species, were excluded from this study.

#### Allelic typing of *MSP-1* gene block 2 for *P. falciparum* isolates

All *P. falciparum* samples were genotyped by amplification of the highly polymorphic regions of *MSP-1* (block 2) using nested PCR as described previously (Snounou *et al*., 1999), with slight modifications for the cycling conditions of the secondary PCR. The oligonucleotide primers sets (Table 1) previously designed by Snounou *et al.* (1999) were used for the detection of the different families MAD20, K1 and RO33 in *MSP-1*. Fragments representing the different alleles were selected and purified using the QIAquick® Gel Extraction Kit (Qiagen, Germany). The MAD20, K1 and RO33 products from nested PCR that showed normal and polymorphic bands were sent for DNA sequencing by using forward and reverse primers.

#### Allelic frequency and multiplicity of infection (MOI)

The prevalence of each allelic type was determined by the presence of PCR products for the type in the total number of amplified bands for the corresponding locus. All positive PCR products were sent for DNA sequencing to avoid false-positive identification. The multiplicity of infection (MOI)
or complexity of infection was calculated by dividing the total number of fragments detected by the number of samples positive for the same marker. Isolates with more than one genotype were considered as polyclonal infection while the presence of a single allele was considered as monoclonal infection.

**Sequencing and phylogenetic analysis of MSP-1 gene block 2 of P. falciparum isolates**

Cycle sequencing was carried out using the ABI PRISM 1 BIGDyeTM terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) in a 3700 DNA Analyzer (Applied Biosystems, USA). Similarity searches were conducted using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1999). DNA sequence data for each allelic family was aligned with other allelic sequences worldwide by using the Clustal W program through Bioedit 5.0.7 software. Phylogenetic analysis was carried out for *P. falciparum* isolates based on MAD20, K1 and RO33 allelic type sequences using UPGMA analysis utilizing the program MEGA 7 (Kumar *et al.*, 2016). To understand the identity of *MSP-1* block 2 allelic types of Malaysian and Thailand isolates with respect to isolates of other regions worldwide, sequence data available in the public domain were downloaded from GenBank. Nucleotide sequences reported in this study are available in the GenBank database under accession numbers KY231851, KT158693-KT158709 for MAD20 family and KY231852, KT158686-KT158692 for K1 family. Nucleotide sequences of RO33 family were not deposited in GenBank because the amplicons were shorter than 200bp.

**RESULTS**

**Allelic diversity of Plasmodium falciparum MSP-1 block 2**

The distribution of *MSP-1* block 2 allelic types in *P. falciparum* isolates from Malaysia and Thailand was determined. A total of 173 (67 Malaysian isolates and 106 Thailand isolates) *P. falciparum* isolates were typed for *MSP-1* block 2 MAD20, K1 and RO33 allelic types by nested PCR. A total of 194 distinct fragments were detected, out of which, 8 and 21 different *MSP-1* alleles were identified in Malaysian and Thailand isolates, respectively. The results showed that 167 (96.5%) blood samples were positive for *MSP-1* block 2 allelic types, while 6 (3.5%) were negative. Comparison of the sequences showed that all the positive samples belonged to one of these three alleles. The overall allelic distribution recorded was higher in MAD20, followed by RO33 and K1. MAD20 was the highest allelic family detected in Malaysian isolates as well as in Thailand isolates followed by RO33 and K1. Of the total blood samples, 74 (42.8%) were positive for only the MAD20 allelic type, 52 (30.0%) for RO33 and 18 (10.4%) for K1 (Table 2). Multiclonal isolates and MOI in Malaysia and Thailand were also given in Table 2. The total multiple clonal infections in Malaysia and Thailand was 13.3% (23/173). Of the 23 samples showing mixed allelic types of infection: 8 (4.6% of overall samples) were K1+MAD20; 5 (3.0%) K1+RO33; 8 (4.6%) MAD20+ RO33 and 2 (1.1%) K1+ MAD20+ RO33 (Table 2). The prevalence of total MAD20, K1 and RO33 allelic types was 53.2% (92/173), 19.1% (33/173) and 38.7% (67/173), respectively, of the studied samples.

Multiplicity of infection (MOI) of Malaysian and Thailand isolates was almost similar which was 1.1 and 1.2, respectively. The length variants of the amplified products were approximately 150-300bp for MAD20, 150-250bp for K1 and 150bp for RO33, only single band was observed for each allelic type. RO33 was observed to be monomorphic with an allele size of 150bp.

**Sequence analysis of Plasmodium falciparum MSP-1 block 2**

A total of 26 different alleles were recognized by sequence analysis of the *MSP-1* block 2 (Figure 3, 4 & 5). In *MSP-1* block 2, the nucleotide and the deduced amino acid sequence were found to be highly polymorphic among the isolates. All the nucleotide changes in these isolates were non-synonymous, as a result, the deduced amino acid variations corresponded to one
or other allele. The MAD20 allele type was more diverse with 17 different alleles (4 alleles from Malaysian isolates and 13 alleles from Thailand isolates) (Figure 3), compared to 7 (1 allele from Malaysian isolates and 6 from Thailand isolates) from the K1 allele type (Figure 4) and two from RO33 allele type (Figure 5).

A limited number of different tripeptide repeat units (5 for MAD20 and 3 for K1) were identified in this study. The sequence variation in MSP-1 block 2 are created by rearrangements of the tripeptide repeat building blocks. In the MAD20 type alleles, the repeat region started with one of five different tripeptide sequences, SKG, SKG, SVA, SVT or SSG, but always terminated with the identical hexapeptide sequence, SVASGG except MAD20 Allele 10 of Thailand isolates which terminated with SGG. The diversity of the MAD20 allele type was caused by differences in these five repetitions (Figure 3). In the K1 type alleles, the tripeptide repeat region always started with SAQ and end with SGT, regardless of differences in the number of tripeptide repeats. Most diversity was due to duplications or deletions of the repeat motifs SAQ, SGP and SGT (Figure 4). RO33 allelic type showed less variation, no tripeptide repeats were observed but non-synonymous nucleotide substitution was seen in Malaysian and Thailand isolates (Figure 5). A change from Gly (GGT) to Asp (GAT) at position 199 was seen in RO33 allelic type from Malaysian and Thailand isolates.

**Phylogenetic analysis of Plasmodium falciparum isolates detected in this study and other regions worldwide based on MAD20, K1 and RO33**

Blast results revealed that 93 to 100% similarity was observed with MAD20 allelic sequences reported for isolates of Philippines, Papua New Guinea, India, Myanmar, Vietnam, China, Cambodia, Ghana, Solomon Islands, Brazil and Malawi. All the allelic families detected in study were compared with isolates of Malaysia, Thailand, Philippines, and other regions worldwide (Table 3). For K1 family, 97-100% similarity was observed with the sequences reported for isolates of Philippines, India, Vietnam, Tanzania, Vietnam, Indonesia, Cambodia, Papua New Guinea and Malawi with Malaysian and Thailand isolates. K1 family seems less diverse compared to MAD20 family. RO33 Allele 1 of Malaysian and Thailand isolates in this study have shown 100% similarity with sequences reported for isolates of Philippines, Thailand and Papua New Guinea. As for RO33 Allele 2, it was identical to sequences obtained from isolates of Tanzania, Malawi and Sudan.

Each phylogenetic tree of MAD20, K1 and RO33 sequences from Malaysian and Thailand isolates were constructed with sequences from other regions worldwide. The phylogenetic of MAD20 allelic type sequences obtained from Malaysian and Thailand isolates showed that all the MAD20 allelic type sequences detected in this study were grouped with MAD20 allelic type sequences from other regions except Malaysia Allele 2 that clustered with Malawi isolate. Malaysia Allele 3 clustered with isolates from China, Thailand and Myanmar with 100% similarity (Figure 6). The phylogenetic tree of K1 allelic type sequences obtained from Malaysian and Thailand isolates showed that K1 allelic type sequences were grouped with K1 allelic type sequences from other regions especially Southeast Asia countries such as Cambodia, Myanmar, Indonesia, Vietnam, Indonesia and Philippines. The isolates of these few countries were closely related and showed less variation (Figure 7). RO33 allelic type sequences obtained from Malaysian and Thailand isolates in this study formed two different groups with RO33 allelic type sequences from other regions (Figure 8). Malaysia and Thailand Allele 1 seems to be more closely related with Asian isolates while Allele 2 showed to group with isolates from African regions.

**DISCUSSION**

Findings showed that the *P. falciparum* isolates in Malaysia and Thailand exhibit extensive genetic polymorphism but showed high similarities. Malaysia MAD20 Allele 3 and Thailand Allele 6 were 100% identical as well as with Myanmar and China isolates. All the allelic families detected in study were
clustered except Malaysia MAD20 Allele 2 which was grouped with Malawi isolates. Unlike findings reported in Lao PDR by Khaminsou et al. (2011), all the K1 allelic type sequences detected in this study were clustered with those reported in different countries, no unique K1 or MAD20 allelic type was identified in this study. Blast analysis revealed that most of the Southeast Asian isolates were highly similar, it was postulated that the same allelic type sequences were circulating in these countries. These findings indicated that the same antimalarial therapy may be effective for the malaria infection in these countries.

The percentage of multiple clonal infections in Malaysian and Thailand isolates was about the same which was ~13.3%. Total multiple clonal infections in this study was much lower than those reported from Myanmar (63.5%) (Kang et al., 2010) and Lao PDR (45.65%) (Khaminsou et al., 2011). Findings of this study showed a low multiplicity of infection (MOI) for both Malaysia (1.1) and Thailand (1.2) reflecting the low intensity of malaria transmission in these areas. The MOI reported in this study was comparable to the previous studies conducted in Malaysia (Atroosh et al., 2011; Mohd Abd Razak et al., 2016) and Thailand (Snounou et al., 1999; Congpuong et al., 2014) but much lower than those reported in areas of high malaria transmission such as Tanzania (Ferreira et al., 1998), Gabon (Aubouy et al., 2003), Brazzaville (Mayengue et al., 2011) and Mauritania (Salem et al., 2014). The MOI of Thai isolate in this study was much more lower compared to three malaria endemic areas in Thailand-Myanmar borders, Tak (MOI=1.89), Ranong (MOI=2.19) and Kanchanaburi (MOI=2.47) provinces (Congpuong et al., 2014), possibly due to high rate of gene flow at the border areas. Moreover, the Thailand samples included in this study were only collected from southeastern coast region. According to Congpuong et al. (2014), high diversity of parasite isolates might affect the treatment outcome. It was evident when declined artesunate-mefloquine combination cure rate was observed in the three provinces (Bustos et al., 2013). A previous study from Iran reported that the emergence of drug-resistant P. falciparum contributed to high level of diversity of MSP-1 along with high proportion of multiclonal isolates (87%) and MOI (3.06) (Zakeri et al., 2005). Recently, malaria situation in Southeast Asian countries has worsen by a newly emerging multidrug resistance P. falciparum strain ‘super malaria’. This so called ‘super malaria’ emerged in Cambodia but had spread rapidly to parts of Thailand, Lao PDR and has arrived in southern Vietnam. It was reported that this strain was resistant to artemisinin and has also evolved to be resistance to piperaquine (Imwong et al., 2017). It is interesting to further monitor the association of parasite diversity with the treatment outcome and drug resistant malaria in these areas. Malaria transmission areas with high MOI need to be vigilant about drug-resistant malaria.

To further investigate the allelic diversity of MSP-1 in P. falciparum isolates from Malaysia and Thailand, sequence analysis was performed. Allelic diversity of MSP-1 block 2 in P. falciparum isolates in this study was due to different numbers of unique tripeptide repeats, which is similar to previous studies (Joshi et al., 2007; Kang et al., 2010). Length variability in MSP-1 families mainly results from repeat sequences. Minor amino acid diversity is created by single-nucleotide replacement. Merozoite surface protein-1 allelic families MAD20, K1 and RO33 showed monomorphic pattern. Monomorphic nature of RO33 family has been reported earlier in isolates of other regions and 150/160bp was the most commonly reported allele in other countries (Konaté et al., 1999; Jordan et al., 2001; Peyerl-Hoffmann et al., 2001; Aubouy et al., 2003). The semi-conserved nature of RO33 family has been reported earlier in isolates of other regions and 150/160bp was the most commonly reported allele in other countries (Konaté et al., 1999; Jordan et al., 2001; Peyerl-Hoffmann et al., 2001; Aubouy et al., 2003). The semi-conserved nature of RO33 had been reported by Bharti et al. (2012) indicating that only 2 types of fragments were observed in Indian isolates. This study showed that extensive genetic polymorphism was observed in P. falciparum isolates in Malaysia and Thailand. There was a total of 26 alleles of MSP-1 block 2, 21 for Thailand isolates and 8 for Malaysian isolates. Sequence analysis of MSP-1 block 2 showed that P. falciparum populations in Thailand have a higher complex genetic diversity.
compared to Malaysian isolates. This might be due to the frequent human migration at Thailand borders which new genotypes were introduced from neighbouring countries. Intragenic recombinations have been reported in contributing to the rich polymorphism among different isolates around the world (Conway and McBride, 1991; Hughes et al., 1992). It was postulated that the high number of alleles of P. falciparum MSP-1 block 2 in Malaysia and Thailand was due to the meiotic recombination events during the sexual stage of parasites that involve genetically distinct clones infecting the same mosquito vector.

In the present study, MAD20 allelic type was found to be the most prevalent in both countries followed by RO33 and K1. These findings are in agreement with previous studies in Thailand (Snounou et al., 1999), Colombia (Gómez et al., 2002), Iran (Zakeri et al., 2005) and Pakistan (Ghanchi et al., 2015) which showed that MAD20 was the predominant allelic family. However, these findings were discordant with studies carried out by Atroosh et al. (2011) and Mohd Abd Razak et al. (2016) reported that RO33 and K1 were the predominant allelic families among P. falciparum in Malaysia. It was postulated that the difference in the distribution of allelic families in P. falciparum isolates in Malaysia was due to factors such as vector population, human host immunity and drug susceptibility pattern of the parasites in the transmission areas (Joshi et al., 2007). Previous studies had reported that MAD20 was the predominant allelic family among P. falciparum population at Thai-Cambodian border (Snounou et al., 1999). However, K1 was reported as the most frequent allele family among P. falciparum population at Thai-Myanmar border (Congpuong et al., 2014) as well as in other countries such as Lao PDR (Kang et al., 2010), India (Bharti et al., 2012), French Guiana (Ariey et al., 1999), Kenya (Takala et al., 2002), Nigeria (Happi et al., 2004) and Peru (Kun et al., 1998). The association between the distribution of allelic families with the severity of malaria has been investigated by previous studies, however, it is not possible to make a definitive conclusion. K1 family was frequently reported in severe cases and RO33 family in asymptomatic malaria cases (Kun et al., 1998; Ntoumi et al., 1995). A previous community-based study in Papua New Guinea reported an association between reduced risk of clinical malaria and infection with parasites of MSP-1 type RO33 (Al-Yaman et al., 1997). On the other hand, previous studies revealed an association between the dominance of K1 allelic family and the existence of asymptomatic malaria infection (Babiker and Walliker, 1997; Amodu et al., 2005). In this study, we could not examine such association as the clinical data of patients was not available. Unfortunately, the demographic data and parasite density of the patients in this study were also not available.

CONCLUSION

The present study reports extensive genetic polymorphisms of P. falciparum isolates in Malaysia and Thailand but high similarities were observed among the isolates based on the MSP-1 gene block 2. Blast analysis revealed that most of the Southeast Asian isolates were highly similar, it was postulated that the same allelic type sequences were circulating in these countries. Results indicated that the same antimalarial therapy may be effective for the malaria infection in these countries.

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Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

YALL, KHC and XTG conceived and designed the experiments. VN, PCL, HCC and IV involved in samples collection and processing. XTG and NJY performed molecular genetic studies and data analysis. XTG, YALL, TCT and KHC drafted the
manuscript. PCL, KHC and YALL contributed the materials, reagents and analysis tools. All authors read and approved the final manuscript.

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