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## The role of the human Duffy antigen receptor for chemokines in malaria susceptibility: current opinions and future treatment prospects

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### Abstract

The Duffy antigen receptor for chemokine (DARC) is a nonspecific receptor for several proinflammatory cytokines. It is homologous to the G-protein chemokine receptor superfamily, which is suggested to function as a scavenger in many inflammatory-and proinflammatory-related diseases. G-protein chemokine receptors are also known to play a critical role in infectious diseases; they are commonly used as entry vehicles by infectious agents. A typical example is the chemokine receptor CCR5 or CXCR4 used by HIV for infecting target cells. In malaria, DARC is considered an essential receptor that mediates the entry of the human and zoonotic malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* into human reticulocytes and erythrocytes, respectively. This process is mediated through interaction with the parasite ligand known as the Duffy binding protein (DBP). Most therapeutic strategies have been focused on blocking the interaction between DBP and DARC by targeting the parasite ligand, while strategies targeting the receptor, DARC, have not been intensively investigated. The rapid increase in drug resistance and the lack of new effective drugs or a vaccine for malaria constitute a major threat and a need for novel therapeutics to combat disease. This review explores strategies that can be used to target the receptor. Inhibitors of DARC, which block DBP–DARC interaction, can potentially provide an effective strategy for preventing malaria caused by *P. vivax*.

### Keywords

*Plasmodium; vivax; knowlesi; DARC; malaria; treatment*

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## Introduction

The Duffy antigen receptor for chemokines (DARC) was first discovered in the 1950s<sup>1</sup> and later defined as a blood group antigen in the late 1960s. It became better known as the cell surface receptor used by the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* to invade the red blood cells (RBCs).<sup>2,3</sup> DARC, a single copy gene located on chromosome 1 (1.q22-1.q23),<sup>4</sup> is a glycosylated transmembrane protein of about 35–40 kDa.<sup>4–6</sup> It is comprised of an extracellular N-terminal domain (containing the chemokine-binding site) and an intracellular C-terminal domain. Alternatively, it is referred to as cluster of differentiation 234 (CD234) or Fy-glycoprotein (Fy).<sup>4</sup> DARC is a minor blood group antigen that has two immunologically distinct and co-dominant alleles referred to as Fy<sup>a</sup> and Fy<sup>b</sup>, which differ by a single base substitution in codon 42 encoding a glycine in Fy<sup>a</sup> and an aspartic acid in Fy<sup>b</sup>.<sup>7</sup> These two alleles result in four major Duffy blood group phenotypes: Fy<sup>a</sup>, Fy<sup>b</sup>, Fy<sup>a+b+</sup>, and Fy<sup>a-b-</sup> (also referred to as Fy-null). Four other less reactive phenotypes, Fy3, Fy4, Fy5, and Fy6, have also been described.<sup>8,9</sup> The Fy-null phenotype results from a Fy<sup>b</sup> gene mutation at amino acid position –46 in the erythroid regulatory element of the DARC promoter region. This mutation blocks promoter activity in cells derived from the hemopoietic lineage by disrupting the binding site for the specific erythroid transcription factor GATA1.<sup>10</sup> This mutation is responsible for the lack of expression of DARC on the surface of erythroid cells. These DARC polymorphisms form the basis for the Duffy blood group.<sup>11,12</sup> The Fy<sup>a</sup> and Fy<sup>b</sup> antigens are common among Caucasians (Fy<sup>a</sup> 66% and Fy<sup>b</sup> 83%) and Asians (Fy<sup>a</sup> 99% and Fy<sup>b</sup> 18.5%) but are far less common in blacks (Fy<sup>a</sup> 10% and Fy<sup>b</sup> 23%). In fact, the Fy<sup>a-b-</sup> phenotype is present in two-thirds of African-American blacks but is very rare in Caucasians.<sup>10,13</sup> Another rare phenotype, Fy<sup>b-</sup>, has also been described among non-Ashkenazi Jews and Brazilian blacks, but its clinical relevance is still unknown.<sup>14</sup>

## DARC as a receptor for chemokines

DARC, also referred to as the Duffy blood group antigen,<sup>15,16</sup> is a promiscuous receptor for several proinflammatory cytokines secreted by immune cells called chemokines, which act as communication signals.<sup>17,18</sup> Most chemokine receptors specifically bind to chemokines of a single class: either the CC or the CXC classes. The DARC binds to chemokines of both the CC and CXC classes, the melanoma growth stimulatory activity (MSG- $\alpha$ /CXCL1), interleukin 8 (CXCL8), regulated upon activation normal T-expressed and secreted (RANTES/CCL5), monocyte chemotactic protein-1 (CCL2), neutrophil activating protein 2 and 3, growth-related gene alpha, epithelial neutrophil activating peptide-78, (CXCL5), and angiogenesis-related platelet factor 1.<sup>19–23</sup> DARC is homologous to the chemokine G-protein chemokine receptors, commonly used as entry vehicles by infectious agents.<sup>4</sup> However, DARC lacks the DRY motif, which is required for G-protein interaction and signal transduction. It is mainly expressed on the surface of erythroid cells and endothelial cells lining post-capillary venules in the kidneys, spleen, and neuronal cells in both Duffy-positive and Duffy-negative individuals.<sup>24,25</sup> DARC is believed to act as a scavenger, reducing excess amounts of toxic chemokines produced in some pathological conditions. It mediates the effects of proinflammatory chemokines on endothelial cells lining post-capillary venules as well as neutrophil emigration to inflammation sites.<sup>24,26–28</sup> Nevertheless, its role as a

scavenger has been questioned and alternatively suggested to mediate chemokine transcytosis leading to apical retention of intact chemokine.<sup>29</sup>

## DARC and associated diseases

As a receptor for many chemokines, DARC has great clinical significance in many disease conditions. It has been implicated as a major actor in some infectious and inflammatory diseases as well as in cancer. DARC negativity has been shown to influence angiogenesis in animals. There is good evidence in mouse and in vitro models for DARC affecting inflammation, in particular leukocyte trafficking, chemokine levels, and malignancy. This suggests that DARC may play a role in the frequent differences in disease outcome seen in African-Americans.<sup>30</sup> It is suggested that the lack of DARC expressivity results in the inability to remove the buildup of angiogenic chemokines, which are believed to contribute to cancer development.<sup>31,32</sup> Consequently, the high rate of prostate cancer in men of African descent has been attributed to the predominance of DARC negativity in this group.<sup>32,33</sup> However, other studies have found little or no correlation between DARC expression on erythrocytes and the risk or progression of prostate cancer in men of African descent.<sup>32,34</sup> DARC has also been implicated in the clinical outcome of other cancers such as breast cancer and metastasis.<sup>35,36</sup> Some other racial differences relying on DARC in endotoxin responses,<sup>37</sup> pregnancy,<sup>11</sup> renal transplantation,<sup>38</sup> and sickle cell<sup>39,40</sup> have also been investigated. In HIV infection, DARC -46C/C is associated with a 40% increase in odds of acquiring HIV-1. Interestingly, regulatory variant genotypes of DARC can influence the course of HIV disease. This gives survival advantage to persons of African ancestry with reduced neutrophil counts<sup>41,42</sup> as well as reduced plasma levels of HIV-1-suppressive and proinflammatory chemokines such as CCL5/RANTES. It is suggested that DARC influences HIV/AIDS susceptibility by mediating the binding of HIV to RBCs and subsequent viral transfer to HIV target cells.<sup>5</sup> On the other hand, some DARC mutations have been found to abrogate receptor expression, leading to a significantly increased susceptibility to HIV-1 infection but, paradoxically, to prolonged survival in HIV-1-infected subjects.<sup>43</sup> Other studies have shown that HIV-1 binds to erythrocytes' DARC, making these RBCs able to transmit HIV to peripheral blood mononuclear cells. Thus, these cells function as a reservoir for HIV-1 or as a receptor for the entry of HIV-1 into CD4 cell subsets, neurons, or endothelial cells. DARC is also clinically relevant in diseases such as asthma and atopy among some populations of African descent,<sup>44</sup> Southeast Asian ovalocytosis,<sup>45</sup> and other inflammation-related diseases.<sup>27,46</sup> Most importantly, DARC plays a critical role in erythrocyte invasion by malaria parasites.

## DARC as a receptor for malaria parasites

The interest on the role of DARC as a scavenger has been diminished slightly, since its establishment as the unique receptor for the human malaria parasite *P. vivax*<sup>2,47,48</sup> and the zoonotic parasite *P. knowlesi*.<sup>3</sup> Evidence for use of DARC for invasion was also reported for the mouse parasite *Plasmodium yoelii*, although another underlying pathway has been identified.<sup>49,50</sup> While *P. knowlesi* only accounts for zoonotic infections in some regions,<sup>51</sup> *P. vivax* is a major public health problem worldwide.<sup>52–54</sup> The World Health Organization reported an estimated 13.8 million cases of clinical illness and 1,400–14,900 deaths due to *P.*

*vivax* malaria in 2015. Historically, *vivax* malaria was regarded as causing a benign and often self-limiting infection. There is increasing evidence of clinical severity of disease with a great pathological and economical burden for inhabitants of endemic regions.<sup>55–57</sup> Similarly, widespread drug resistance has also been reported.<sup>58–60</sup>

Erythrocyte invasion by malaria parasites is essential for blood-stage development. This invasion process is mediated by specific ligand–receptor interactions between the parasite (merozoites) and the host erythrocytes.<sup>61–63</sup> It is believed that the *P. vivax* Duffy binding protein (PvDBP) and the *P. knowlesi* Duffy binding protein alpha on the merozoite surface interact with DARC on the reticulocyte surface precipitating the junction formation step necessary for invasion.<sup>3,62,63</sup> Historically, the vital need of the Duffy binding protein (DBP)–DARC interaction was evident from the virtual absence of *P. vivax* malaria in populations with a high prevalence of DARC negativity<sup>2,64,65</sup> and the refractoriness of *P. knowlesi* merozoites to invade Duffy-negative human erythrocytes.<sup>3</sup> This is an indication that the absence of DARC on the erythrocyte surface has a protective advantage against *vivax* and *knowlesi* malaria. More additional compelling evidence of the importance of the DBP–DARC interaction was demonstrated by the protective effect against clinical *vivax* malaria by the Fy<sup>a</sup> allele.<sup>66</sup> The vital need of this DBP–DARC interaction during reticulocyte invasion makes DBP a prime target for vaccine-mediated immunity against malaria caused by the parasites.

Variations in the *Fy* gene have been associated with phenotypic variation in susceptibility to malaria. It has been demonstrated that adherence of the DBP ligand domain (DBPII) to erythrocytes is significantly reduced for erythrocytes in heterozygous individuals carrying one Duffy antigen-negative allele.<sup>67</sup> Individuals with the Fy<sup>a</sup> phenotype demonstrated a 30%–80% reduced risk of clinical *vivax* but not *falciparum* malaria in a prospective cohort study in the Brazilian Amazon.<sup>66</sup> The Fy<sup>a</sup> allele has reached fixation in Southeast Asian populations, areas thought to be the wellspring of *P. vivax*.<sup>68</sup> Conversely, Fy<sup>b</sup> is present in North and Northern-central European populations and admixed in many populations with strong Northern European influence. This distribution of *Fy* alleles suggests a selective advantage against *P. vivax* malaria.<sup>66</sup> Importantly, inhibitory antibodies to the DBP ligand domain were much more effective in blocking DBP binding to erythrocytes expressing Fy<sup>a</sup> compared with Fy<sup>b</sup>. This suggests that the relative frequencies of Fy<sup>a</sup> and Fy<sup>b</sup> alleles in these populations may affect DBP vaccine efficacy.

Contrary to the established DBP–DARC invasion pathway, there is increasing evidence of a DARC-independent invasion of human reticulocytes by *P. vivax*.<sup>69–73</sup> In Madagascar, with a mixture of Duffy-positive (Fy+) and -negative (Fy–) populations of diverse ethnic backgrounds, there was a significant reduction in the prevalence of clinical *vivax* malaria in Duffy-negative individuals compared with Duffy-positive individuals.<sup>70</sup> Similarly in the Brazilian Amazon, two cases of clinical *vivax* malaria were observed in samples of Duffy-negative individuals obtained from Rondônia.<sup>71</sup> It is not yet clear if these historically anomalous cases represent random isolated events that have always occurred or a new phenomenon related to *P. vivax* evolving to use an alternate DARC-independent pathway for invasion. Alternatively, DBP might remain the critical invasion ligand using alternate receptors for invasion. A *P. vivax* DBP homolog erythrocyte binding protein has been

identified. This novel ligand is anticipated to be involved in an alternate invasion pathway to DBP.<sup>74,75</sup> However, the exact role of this molecule in the invasion of Duffy-negative individuals is still unknown. A recent study suggested that an unusual DNA expansion of DBP in two Duffy-negative *P. vivax* infections studied suggests that an expansion of DBP may have been selected to allow low-affinity binding to another receptor on Duffy-null erythrocytes.<sup>76</sup> Nevertheless, no other receptor other than DARC has been described for *P. vivax*.

Several in vitro assays have been used to study the interaction between the ligand domains of *P. knowlesi* and *P. vivax* DBPs. DBP binds to DARC on host reticulocytes through a conserved cysteine-rich Duffy binding-like (DBL) domain known as region II (DBPII), which is characterized by 12 conserved cysteine residues. The region between cysteines 4 and 7 constitutes the major determinants for receptor recognition.<sup>77–80</sup> The binding sites for these two different parasite molecules map to a 35 amino acid region at the N-terminal extracellular domain (ECD1) of DARC.<sup>81</sup> A peptide consisting of these 35 amino acid residues as well as a recombinant protein consisting of the N-terminal 60 residues of DARC (nDARC<sub>1–60</sub>) blocked PvDBP binding to human reticulocytes.<sup>81,82</sup> Two tyrosine molecules within the 35 amino acid region (Tyr30 and Tyr41) are post-translationally sulfated, with sulfonation of Tyr41 associated with high-affinity binding of *P. vivax* DBPII. Specifically, a sulfated recombinant DARC N-terminus construct inhibits the DBPII erythrocyte interaction to a greater extent than an unsulfated construct.<sup>82</sup> However, a recent crystal structure of DBPII in contact with nDARC did not show any contact between DBPII and Tyr41 of DARC.<sup>83</sup> This suggests that the modification did not directly facilitate DARC–DBPII interaction but probably modified the presentation of DARC or its exposure. Tyrosine sulfation is also critical in the association between the HIV gp120 and the N-terminal domain of CCR5, a receptor for most HIV-1 isolates for invasion.<sup>84,85</sup> Similar to DBP, a tyrosine-sulfated peptide based on the amino terminus of CCR5 specifically blocks HIV-1 entry and gp120 association with CCR5.<sup>86</sup>

The molecular and structural basis of DBP–DARC interaction was recently resolved.<sup>83,87</sup> This structure reveals that DARC recognition by DBP is through a receptor-mediated ligand dimerization (Figure 1). This mechanism of interaction suggests that dimerization is critical for, and driven by DARC binding, leading to the formation of a stable high-affinity complex composed of two DBP and two DARC molecules.<sup>87</sup> This complex is then believed to precipitate junction formation to initiate entry into the host cell. During this process, DBP dimerization creates a pocket (DARC binding pocket), which fits the N-terminus of DARC. This phenomenon is similar to receptor recognition in DBL domains of other members of the DBL superfamily such as PfEMP1, VAR2CSA, and PfEBA-175.<sup>88,89</sup> The specific molecular interactions at the DBP–DARC interface were analyzed, revealing critical contact residues within the N-terminal extracellular domain of DARC. Most importantly is the DARC binding pocket, which has a strong affinity for a sulfotyrosine of DARC.<sup>82,83</sup> Key residues for this interaction were located within subdomain 2 of DBPII, with residues F261–T266, L270–K289, and Q356–K367 forming critical contacts with the DARC extracellular domain.<sup>83</sup> The residues that play a critical role in this interaction are important targets of protective immunity. Unlike the parasite ligands, ECD1 alone is not sufficient for chemokine

binding. The close association of the four extracellular domains of DARC (ECD1–ECD4) through disulfide bonds is required to create an active chemokine-binding pocket.

## Current opinion and future therapeutic prospects

Chemokines and their receptors play a major role in facilitating the entry and transmission of intracellular pathogens, typical examples being in HIV and *P. vivax* infections. As such, they represent attractive targets for novel therapeutics.<sup>90–92</sup> In order to exploit the potential of these chemokine receptors as drug targets, there is a need to identify the specific ligands and receptors that are rate limiting in a given disease setting and develop reagents to block their interaction. Methods that can prevent receptor–ligand interaction such as interfering with signaling pathways that are induced upon receptor activation and modification of receptor trafficking pathways can be explored to develop therapeutics.

In *P. vivax* and *P. knowlesi*, until other parasite proteins and their subsequent unknown receptors are identified, the sole dependence on DARC makes this outstanding nonspecific multireceptor for chemokines an invaluable target to explore procedures to inhibit blood-stage propagation of *P. vivax*. Extensive effort has been put into designing therapeutic strategies focused on the interaction between DBPII and DARC. This is mainly based on the evidence that some individuals are able to develop long-lasting and strain-transcending inhibitory antibodies against DBPII. Notwithstanding, strategies targeting the other counterpart of this host–parasite interaction in the blood stream, that is DARC, have not been intensively explored. To date, it is mostly established that *P. vivax* and *P. knowlesi* invasion depends on the parasite Duffy binding protein DBL domain (PvDBPII or *P. knowlesi* Duffy binding protein alpha) engaging DARC on RBCs. Inhibition of this key interaction provides an excellent opportunity for parasite inhibition. Individuals in endemic regions produce anti-DBP antibodies capable of blocking DBP–DARC interaction and parasite invasion of reticulocytes.<sup>93–96</sup> Epitopes that are targets of these neutralizing antibodies mapped to the dimer interface, DARC binding pocket and surround the DARC binding pocket,<sup>87,97</sup> suggesting that these protective antibodies target DBP functional regions, and interfere with dimerization or prevent receptor binding. This shows the importance of the DARC binding pocket and dimer formation for parasite survival. The structural determinants for DBP–DARC interaction have been identified.<sup>83,87</sup> These regions, especially the DARC epitopes that make contact with DBP, represent critical regions that can be exploited for rational design of potent neutralizing therapeutics aimed at disrupting erythrocyte binding. Specific examples include designing vaccines and small molecule inhibitors that can compete with DBP for binding to DARC. These molecules if targeted to the dimer interface and receptor-binding pocket, they could prevent dimer formation and consequently merozoite invasion. Targeting DBP–DARC is effective against *P. vivax*, as natural selection of a Duffy-null phenotype has largely eliminated *P. vivax* in West Africa.<sup>2</sup> As receptor-mediated ligand dimerization is a general mechanism by which EBL proteins engage receptors, disrupting dimerization could be a viable strategy for therapeutic intervention against other *Plasmodium* species.

## Vaccines as a therapeutic tool

Vaccines are considered the best means of control of infectious diseases. Over 70 different vaccines against *P. falciparum* are in development, and ~23 are currently undergoing clinical trials. The most promising vaccine against *P. falciparum*, RTS,S/AS01, has completed Phase 3 clinical trials. Unfortunately, the same advances have not been seen for vaccines against *P. vivax*. A series of *P. vivax* merozoite antigens that offer great potential as vaccine candidates has been identified and immunologically characterized. These include the apical membrane antigen (AMA-1),<sup>98</sup> DBP,<sup>99,100</sup> reticulocyte binding proteins,<sup>101,102</sup> and merozoite surface proteins.<sup>103–105</sup> However, the development of a *P. vivax* vaccine has been hampered by technical difficulties. *P. vivax* preferentially invades reticulocytes, which account for only 1%–2% of total peripheral blood circulation. Because of the difficulty in obtaining enough reticulocytes, long-term culture for *P. vivax* has been a major challenge and a drawback to adequately study the biology of the parasite. Despite these challenges, a number of potential strategies are being explored for the development of a *P. vivax malaria* vaccine.<sup>106–109</sup>

DBP is a leading vaccine candidate for blood-stage *P. vivax* malaria. Individuals in endemic regions produce anti-DBP antibodies, which block DARC binding and reticulocyte invasion.<sup>93,96</sup> However, this antibody response is generally weak and bias toward the development of strain-specific immunity.<sup>95,97,100</sup> Despite this, a few elite responders are able to produce strain-transcending inhibitory antibody responses,<sup>96,110</sup> suggesting the existence of conserved neutralizing epitopes on DBP. Some studies have reported strategies to overcoming strain-specific immunity in *P. vivax*, including a combination vaccine involving multiple-variant DBP alleles<sup>109</sup> or a synthetic DBP antigen devoid of the dominant polymorphic B-cell epitopes.<sup>108,111</sup> These studies, which are aimed at focusing on immune response to functional conserved neutralizing epitopes on DBP, could be optimized to target especially those residues that make contact with the DARC binding pocket. Notwithstanding, the efficacy of a DBP-based vaccine may differ among populations with varying Fy phenotypes.

## Gene knockouts and targeted gene mutagenesis

One of the greatest ways to validate a target is to study the effect of the deletion of the gene of interest in vivo (receptor “knock out”). Several chemokine receptors have been validated as successful targets of anti-inflammatory therapies and anti-HIV infectivity strategy. CCR5 is the best example as a target, with a natural gene “knock out”. A natural deletion of a 32 base pair region on an allele of CCR5 (Δ32-CCR5) results in a lack of surface expression of CCR5. While individuals homozygous for the Δ32-CCR5 are resistant to HIV infection, those heterozygous for the deletion develop a delayed progression to disease.<sup>112</sup> Interestingly, individuals who are homozygote for the Δ32-CCR5 mutation do not appear to have any adverse health issues. A long-standing goal of biomedical research is to develop efficient and reliable ways to make precise, targeted changes to the genome of living cells similar to the natural Δ32-CCR5 mutation. A new biotechnology tool for genome editing, CRISPR, has gained wide interest in the scientific world and researchers believe that it could transform the field of biology.<sup>113</sup> CRISPR allows the ability to edit genomes with unprecedented precision, efficiency, and flexibility. An example is engineered monkeys with

targeted mutations to prevent HIV infections in human cells.<sup>114</sup> However, this application is limited because it provides only temporary inhibition of gene function and unpredictable off-target effects.<sup>115</sup> Considering the fact that natural Duffy negativity does not create any adverse health problems to humans, this technology can be exploited to delete the Duffy positivity footprint in peripheral blood erythrocytes. Developing a strategy to target DARC expression in stem cells could be an interesting way to limit DARC in peripheral blood erythrocytes, without necessarily affecting DARC expression in endothelial cells.

An alternate DARC-related approach to vaccines is the use of novel agents specific for individual malaria species targeting their pathways of invasion. A typical example includes CCR5 blockers, already in use for HIV treatment, which could potentially block DARC, therefore inhibiting invasion of the target cell.<sup>116,117</sup> Likewise, many studies have suggested artificial aberration of the host pathway by target mutagenesis of either RBC receptors to abolish or reduce susceptibility of the host to malaria. Zinc finger array precursors of zinc finger nucleases, which are artificial hybrid restriction enzymes, are becoming powerful tools for primary editing of host genomes as a strategy to halt pathogen infectivity. This strategy has been established in HIV-1 resistance in CD4+ T cells by disrupting the coding region of CCR5 upstream of the  $\Delta 32$  mutation.<sup>118,119</sup> Similarly, with appropriate optimization in vitro to enhance specificity to DARC, this strategy could potentially be applied to the development of an experimental gene-based Malaria vaccine.<sup>120</sup> Alternatively, meganucleases and transcription activator-like effector nucleases<sup>121</sup> that recognize longer stretches of DARC and DNA, especially the N-terminal region, may serve the specific purpose of abrogating invasion of RBCs by *P. vivax*. The feasibility of this approach is supported by existing evidence pointing to resistance of RBCs of naturally selected Duffy-negative blacks to *P. vivax* infection<sup>10</sup> and the fact that DARC negativity will have no adverse effect on human health.

## Small molecule agonists and antagonists as inhibitors of receptor-ligand interaction

The interaction between chemokine receptors and their ligands involves initial interaction with the N-terminal extracellular domains of the receptor, generally mediated by electrostatic forces. Modifications of these regions, using chemokine analogues, either truncations or extension of the amino terminus,<sup>122–124</sup> have been found to retain affinity for the receptors, while impairing signaling. Chemokine receptor antagonists are still in early stages of development.<sup>125</sup> Clinical trials using chemokine receptor antagonist have been reported for many diseases. Yet, the most advanced studies have been performed with CCR5 inhibitors used as retrovirals against HIV. Through screening of small molecules, Pfizer was able to identify a small molecule inhibitor that could block the gp120 binding to the chemokine receptor CCR5.<sup>126</sup> The sites bound by most of these analogues are not yet identified. However, TAK-779, an antagonist of CCR5 with potent anti-HIV activity, binds to a cavity formed between helices located near the extracellular surface of the receptor, which is different from the extracellular loop known as the ligand binding site.<sup>116,117</sup> Many other studies have reported high-affinity antagonists for a series of chemokine receptors, including CCR1, 2, and 5 and CXCR 2 and 4.<sup>117,127,128</sup>



The biology of DARC is very closely related to that of the HIV co-receptors CXCR4 and CCR5. Dimerization following ligand activation has been reported for DARC as well as CCR2, CCR5, and CXCR4.<sup>87,129,130</sup> Considering the similarities in the interactions between antagonists and receptors within the G-protein-coupled receptor superfamily, antagonists/inhibitors, that have been developed for other members will help the rational design of agonists and antagonists of chemokine receptors such as DARC to prevent *P. vivax* malaria. A similar strategy aimed at identifying small molecule inhibitors of DARC, especially the extracellular N-terminal region bound by DBP, will be greatly useful in preventing *P. vivax* infection of human reticulocytes. In the absence of a continuous culture system for *P. vivax*, short-term in vitro *vivax* cultures<sup>131</sup> and standard in vitro assays such as COS 7<sup>80</sup> and flow cytometry-based binding assays<sup>132</sup> as well as the nDARC assay<sup>82</sup> could serve as useful surrogates to screen small molecule libraries to identify potential inhibitory molecules to DBP–DARC interaction. It has been shown that chemokines such as CXCL1 and CXCL8, DBP, and anti-Fy6, a DARC antibody, all bind to similar molecular determinants on DARC and are able to block DBP–DARC interaction by *P. knowlesi*.<sup>15,81,133,134</sup> This suggests that molecules, which compete with DARC for binding, could be used as therapeutics for *vivax* malaria. Novel small molecule inhibitors of DARC such as monoclonal antibodies, soluble receptors, or variant versions of the protein, specifically targeting the DARC binding pocket, can be exploited. This could serve as potential effective strategy for antimalarial therapy either alone or in combination with existing antimalarial drugs to develop new drugs to block DBP–DARC interaction, thereby preventing reticulocyte invasion and consequently *P. vivax* malaria.<sup>135</sup> It is generally suggested that for G-protein-coupled receptors, small molecule agonists or antagonists may exert their effects by stabilizing either an active or a non-active form of the receptor, rather than simply blocking the physical interaction between the receptor and the ligand.<sup>136</sup>

## Conclusion

Emerging resistance of *P. vivax* to current antimalarial drugs demonstrates an urgent need to develop new and alternative approaches to prevent this widespread cause of malaria. Inhibitors of DARC can serve as potential effective strategy for preventing malaria caused by *P. vivax*. Blocking DARC with small molecule inhibitors is a viable, attractive, and increasingly potential new therapeutic approach to prevent *vivax*-induced malaria.<sup>137</sup> Historically, it has been demonstrated that populations in West Africa do not express DARC on their erythrocytes and as such are resistant to *vivax* malaria.<sup>2</sup> In HIV infection, a 32 pb deletion (–32) in the coding sequence of CCR5, the co-receptor for HIV, inhibits expression of this receptor on HIV target cells, thereby preventing HIV infection. Despite the presence of DARC-negative as well as CCR5-negative phenotypes in such individuals, these genetic deficiencies do not seem to play any adverse physiological effects on these individuals. This suggests that blocking DARC will not lead to any deleterious consequences in humans. The parallels of the –32-CCR5 mutation as a protective factor in HIV infection and DARC negativity as a protective factor in *P. vivax* infection indicate that any inhibitors of DARC will be effective in preventing *P. vivax* malaria.

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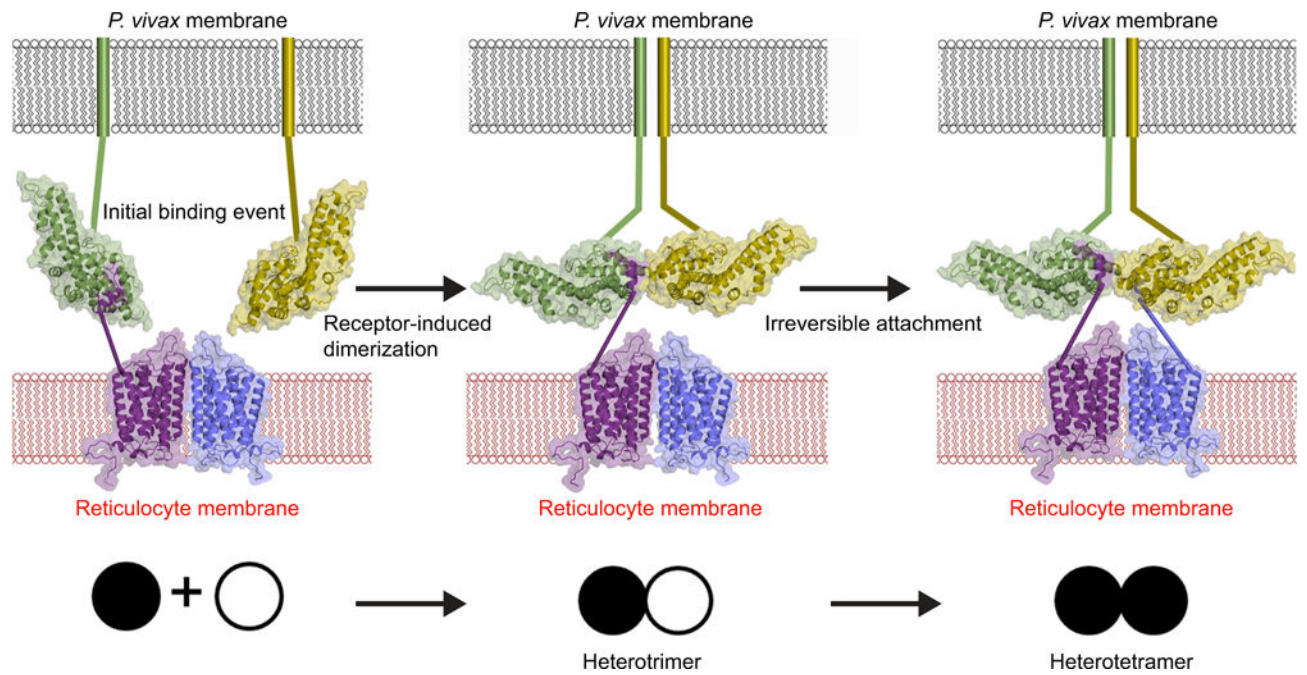
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**Figure 1. A model for DBP–DARC interaction during invasion**

**Notes:** DBP binds to DARC via a model of receptor-mediated ligand dimerization.

Dimerization of DBP is induced upon receptor binding and drives recognition of DARC. An initial binding event is followed by receptor-induced dimerization (DBP–DARC heterotrimer). This brings a second DBP molecule in close proximity to a second DARC ectodomain in the DARC homodimer. A second binding event creates the DBP–DARC heterotetramer. DBP molecules: green and yellow. DARC19–30 molecules: purple and blue. A schematic for the stepwise assembly is shown at the bottom. Closed circle: bound DBP and open circle: unbound DBP. Adapted from Batchelor JD, Malpede BM, Omattage NS, DeKoster GT, Henzler-Wildman KA, Tolia NH. Red blood cell invasion by *Plasmodium vivax*: structural basis for DBP engagement of DARC. *PLoS Pathog.* 2014;10(1):e1003869.<sup>83</sup>

**Abbreviations:** DBP, Duffy binding protein; DARC, Duffy antigen receptor for chemokine; *P. vivax*, *Plasmodium vivax*.