



## Review article

# P-glycoprotein: new insights into structure, physiological function, regulation and alterations in disease

Iman Imtiyaz Ahmed Juvale<sup>a</sup>, Azzmer Azzar Abdul Hamid<sup>b</sup>, Khairul Bariyyah Abd Halim<sup>c</sup>, Ahmad Tarmizi Che Has<sup>a,\*</sup><sup>a</sup> Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia Health Campus, Kubang Kerian, Kota Bharu, 16150, Kelantan, Malaysia<sup>b</sup> Department of Biotechnology, Kulliyah of Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200, Kuantan, Pahang, Malaysia<sup>c</sup> Research Unit for Bioinformatics and Computational Biology (RUBIC), Kulliyah of Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200, Kuantan, Pahang, Malaysia

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## ABSTRACT

The multidrug resistance phenomenon presents a major threat to the pharmaceutical industry. This resistance is a common occurrence in several diseases and is mediated by multidrug transporters that actively pump substances out of the cell and away from their target regions. The most well-known multidrug transporter is the P-glycoprotein transporter. The binding sites within P-glycoprotein can accommodate a variety of compounds with diverse structures. Hence, numerous drugs are P-glycoprotein substrates, with new ones being identified every day. For many years, the mechanisms of action of P-glycoprotein have been shrouded in mystery, and scientists have only recently been able to elucidate certain structural and functional aspects of this protein. Although P-glycoprotein is highly implicated in multidrug resistant diseases, this transporter also performs various physiological roles in the human body and is expressed in several tissues, including the brain, kidneys, liver, gastrointestinal tract, testis, and placenta. The expression levels of P-glycoprotein are regulated by different enzymes, inflammatory mediators and transcription factors; alterations in which can result in the generation of a disease phenotype. This review details the discovery, the recently proposed structure and the regulatory functions of P-glycoprotein, as well as the crucial role it plays in health and disease.

## 1. Introduction

Membrane protein transporters serve a crucial biological function by removing toxic substances from the cytosol and facilitating the uptake of essential nutrients into the cell. This protective function is vital for the survival of all living organisms. A subset of these embedded proteins, the multidrug transporters are important transmembrane glycoproteins that actively transport small lipophilic molecules out of the cell against their concentration gradients [1]. Numerous studies have highlighted the important role of multidrug transporters in drug absorption, distribution, metabolism and elimination [2]. The most well-known multidrug transporters belong to the ATP-binding cassette (ABC, e.g., ABCB1, ABCG2) and solute carrier (SLC, e.g., SLC22A6, SLC22A8) families [3]. Alterations in these drug transporters because of genetic mutations, drug-drug interactions or environmental factors have been shown to

result in toxicity [4]. Several clinical and laboratory studies have revealed drastic changes in serum and tissue drug levels because of drug-transporter interactions [2]. Because of multidrug transporters, the degree of drug efficacy can vary from patient to patient. A dose that might be effective for one individual may be toxic for another individual and it is also highly possible that the drug may have no effect at all. One of the most difficult obstacles faced by the pharmaceutical industry in recent years has been the development of drug resistance. This resistance, which can occur in several diseases, including human immunodeficiency virus (HIV) [5]; cancer [6]; nervous system disorders, such as schizophrenia, epilepsy and amyotrophic lateral sclerosis (ALS) [7, 8, 9]; systemic autoimmune disorders, such as lupus erythematosus (LE) and rheumatoid arthritis (RA) [10, 11]; and inflammatory pain [12, 13] has been largely attributed to the multidrug transporter protein, P-glycoprotein (PGP).

\* Corresponding author.

E-mail address: [ahmادتarmizi@usm.my](mailto:ahmادتarmizi@usm.my) (A.T. Che Has).

## 2. P-glycoprotein: an overview

Burchenal and colleagues were the first to report a case of drug resistance, which occurred with 4-amino- $N^{10}$ -methyl-pteroylglutamic acid in a mouse model of leukaemia [14]. A few years later, two other groups also observed a similar type of resistance to the antibiotic actinomycin D in HeLa cells [15] and Chinese Hamster Ovary (CHO) cells [16]. Thus, the idea of multiple drug resistance (MDR) emerged [16], and this concept was later fully elucidated when the ATP-dependent efflux of another antibiotic, daunomycin, was observed in resistant Ehrlich ascites carcinoma cells that also showed cross resistance to vinca alkaloids, which are naturally occurring plant compounds of unrelated structure and function [17]. Surface labelling studies in CHO cells that demonstrated resistance to colchicine, as well as cross resistance to a variety of amphiphilic compounds, resulted in the discovery of a 170,000 Da cell-membrane efflux pump [18]. This pump was given the name PGP, or the permeability glycoprotein, because of its ability to alter the rate of drug permeation; the amount of PGP expression being correlated with the extent of drug resistance. This MDR phenotype was further investigated at a genetic level and following several cloning studies in human, as well as animal cell lines, the ABCB1 gene was shown to be responsible for generating the multidrug transporter, PGP [19, 20, 21, 22, 23].

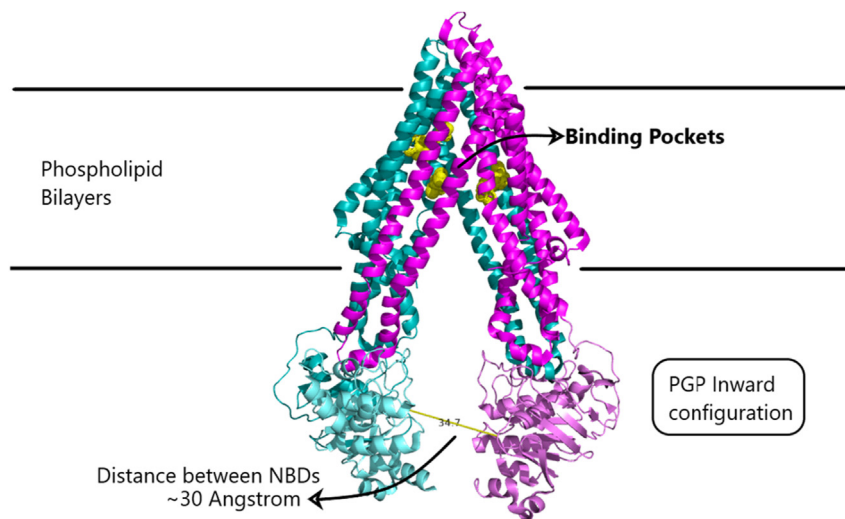
PGP or multidrug resistance protein-1 is a transmembrane unidirectional efflux pump that uses ATP to actively transport substances out of the cell against their concentration gradients [24]. This efflux function of PGP has been observed in several micro-organisms and similar transporters are implicated in cases of antibiotic resistance [25]. In healthy tissue, PGP controls the rate of cellular uptake of foreign substances, their distribution, as well as their elimination thus possessing the ability to directly influence absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of pharmaceutical drugs, affecting both efficacy and bioavailability [26]. ADMET properties always need to be taken into consideration when developing a new drug as they are crucial for determining its dosage, toxicity, route, and frequency of administration. PGP is known to bind to a wide range of substrates that are structurally varied [26, 27], hence, although a drug may seem promising in the early stages, an efflux pump like PGP can reduce its efficacy by pumping the drug out of the cell and away from the target site, resulting in drug resistance. Therefore, the Food and Drug Administration (FDA) encourages screening during the early stages of drug development to check for possible PGP substrates [27]. This helps improve the success rates during the later stages of drug development and also lowers financial costs [27]. However, the screening process can be challenging since different laboratories report conflicting results [27], further causing a setback in the drug developmental process.

In nature, PGP substrates are known to be lipophilic, basic nitrogen-containing compounds that can form several hydrogen bonds [28]. Known PGP substrates include various antineoplastic agents [29, 30, 31], antiepileptic drugs [32, 33, 34],  $\beta$ -adrenoceptor antagonists [35, 36], calcium channel blockers [37, 38], steroids [39, 40, 41], opioids [42, 43, 44], immunosuppressive drugs [45, 46], HIV protease inhibitors [47, 48, 49, 50], antiemetics [51, 52], anthelmintics [53, 54, 55], antibiotics [56, 57, 58], lipid-lowering agents [59, 60] and histamine H1 receptor antagonists [61, 62], with up to 480 substrates identified so far and the number still rising [63]. As such, there is an urgent need to suppress PGP function to improve drug efficacy in MDR diseases by: either lowering or inhibiting the PGP expression levels; blocking the ATPase enzyme activity that is usually responsible for providing PGP with the energy for efflux; or preventing the interaction between PGP and the drug of interest [64]. However, to achieve any of these goals it is important to have an in-depth knowledge of the structure, regulation and physiological functions of PGP to develop appropriate drugs to modulate MDR.

## 3. The structure of P-glycoprotein

Located in the phospholipid bilayer, the PGP pump comprises of two homologous transmembrane domains (TMDs), each containing six membrane-spanning  $\alpha$ -helices and one cytoplasmic nucleotide binding domain (NBD) [65] (Figure 1). The two TMDs are linked together by a flexible 75-amino acid protein containing several phosphorylation sites [66]. X-ray crystallography of the inward facing conformation of PGP revealed a 6000  $\text{\AA}^3$  internal cavity and a 70–200  $\text{\AA}^3$  pore on the extracellular side, with the NBDs separated by 30  $\text{\AA}$  [67] (Figure 1). The internal cavity is wrapped by 12  $\alpha$ -helices that extend from the centre of the phospholipid bilayer to the cytoplasm [68]. The inward facing conformation of PGP also has various binding pockets containing both aromatic and hydrophobic residues along with amino acids with polar side chains such as Q343, Q721, Q942, Q986, and S975 (Figure 1). This arrangement enables the formation of both van der Waals and hydrogen bonds, thus allowing a wide array of substrates to interact with the protein [69, 70]. PGP has been shown to interact with various stereoisomers of the same compound [71]. The tertiary structure of PGP provides enhanced flexibility with three-dimensional reorientation ability, which further facilitates its interaction with a wide range of substrates [72]. Unlike proteins that bind according to the induced-fit model or the lock-and-key model, PGP does not have a fixed ligand-binding pocket [73, 74]. Therefore, polar, non-polar, linear, hydrophobic, and aromatic compounds with varying molecular weights ranging from 250 to 4000 Da have all been identified as substrates of PGP [75]. As with all ABC proteins, each NBD is made up of two Walker regions, regions A (GXGKST; X is a non-specific amino acid) and B (DEATSALD). The difference between proteins that are ABC transporters and non-transporters lies in the position of the signature conserved motif, C (LSGGQ), which in the case of ABC transporters is present between A and B [76, 77, 78] (Figure 2). These three motifs are essential for forming the two active sites for ATPases, each site consisting of a Walker A and B motif from one NBD and a C motif from the other [79]. As the cytosolic concentration of ATP is much higher, around 1–10 mM, compared to that of the domain binding constant (0.01mM), both the ATP-binding sites are present on the intracellular side [79, 80]. Even though the inward-facing conformation is more energetically feasible, the PGP pump usually exists in the outward-facing conformation [81]. Each of these sites contains a highly conserved glutamate residue that functions as a catalytic base for the hydrolysis of ATP. Several studies have shown that alterations in any one of these glutamate sites can diminish the ATPase activity, whereas mutations at both the sites fixes the PGP structure in an occluded nucleotide conformation [82, 83, 84].

The switch from the inward- to the outward-facing conformation involves the tilting and rotating of both the TMDs resulting in the contraction of the cytoplasm-facing inner cavity, thus allowing the efflux of several substrates out of the cell [85] (Figure 3). In the outward conformation, the NBDs dimerise in a head-to-tail formation, with each interface being bound to an ATP molecule to stabilise the structure [79]. This outward-facing conformation is achieved when transmembrane region (TM) 4 and 5 of the first TMD pivot inwards along with TM10 and 11 of the second TMD. The structure is further stabilised by the outward pulling action of TM7 and 8 away from TM9 to 12 [79]. Another important element involved in this structural switch is the residue that lies between the NBD and TMD interfaces, called the Q-loop. The Q-loop (Q475 of the NBD1 and the Q1118 of the NBD2) has been shown to increase the van der Waals forces with the C motif of the other NBD by using a magnesium ion and a  $\gamma$ -phosphate for ATP binding [79, 85, 86]. In addition to the Q-loop, several intracellular helices (IH), including IH1 and 2, and other TM helices undergo changes because of the van der Waals forces between the  $\alpha$ 3-Q-loop and the coupling helices [85]. These changes in structure involve the formation of bonds between TM6 and TM4, and TM6 and TM5, and the dissociation of the bonds between TM1 and TM6. The coiling of the TM3 and TM6 regions results in the



**Figure 1.** Three-dimensional structure of P-glycoprotein (PGP) transporter. The active binding pockets of PGP in the inward configuration with separated nucleotide binding domains and the pockets are represented as yellow spheres and the distance between NBD is shown by yellow dots.

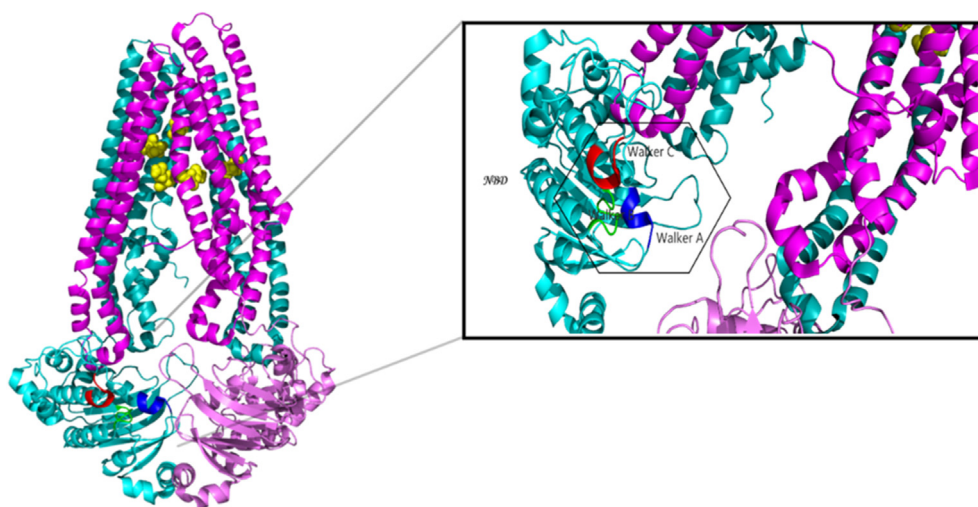
contraction of the inner cavity, and the opening of the extracellular gates [85]. Compared with the unwound form in the inward-facing conformation, TM4 also acquires a more coiled,  $\alpha$ -helical structure [85]. This outward-facing conformation is further stabilised by a Glu620-Arg644 interaction until the substrates are expelled [85]. To date, no significant structural differences in the NBD-TMD interfaces have been observed during the switch from the inward- to the outward-facing conformation, suggesting that this entire region functions as a singular body during drug transport [66, 79]. Even in the outward-facing conformation, the extracellular gate is extremely narrow, which ensures that substrates are actively transported out of the cell in a unidirectional manner [79, 85].

This switch from the inward- to the outward-facing orientation results in a redistribution of the drug-binding residues with more of these binding regions facing the extracellular side [79]. ATP plays an essential role in the stabilisation of this conformation and several studies have suggested an association between the ATPase activity of PGP and substrate efflux leading to drug resistance [87, 88, 89, 90, 91]. It has been reported that PGP can have lower drug affinity in the presence of ATP [92, 93, 94, 95, 96], while the use of the ATP analogue,

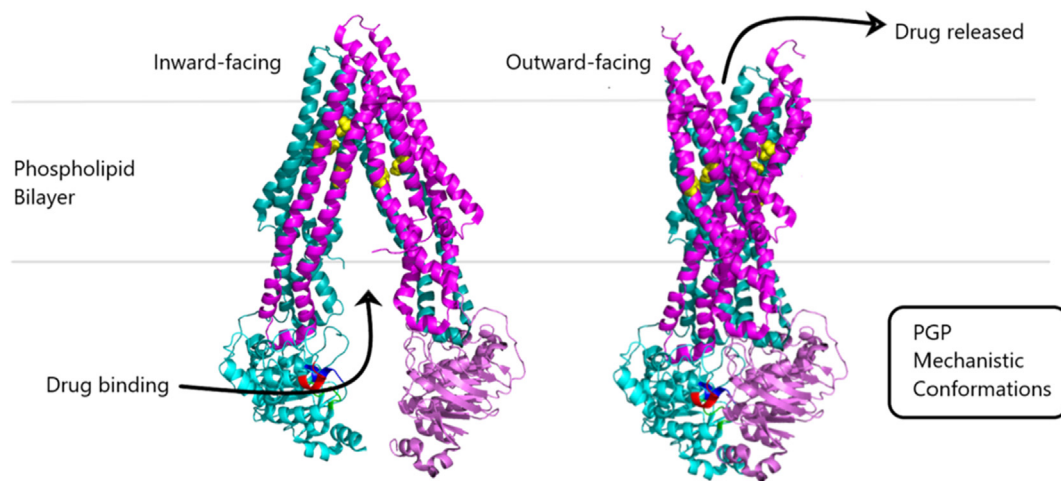
adenylylimodiphosphate, results in drug efflux [97]. These results suggest that while the binding of ATP facilitates the outward-facing conformation of PGP, it is the efflux of the drugs that results in ATP hydrolysis [79]. Although PGP has two active sites for ATPase activity, only one ATP molecule has been observed to be hydrolysed at a time [98]. Other nucleotide trapping experiments have further corroborated this data with activity being detected at only one catalytic site [99, 100], indicating structural asymmetry of the two ATPase sites in the outward-facing conformation. These results also suggest that ATP hydrolysis at any one of these sites is enough to destabilise the structure [79]. This destabilisation results in the potential energy stored within the coiled TM3 and TM6 regions being converted into an elastic spring-like force resulting in a rapid reversal of the outward-facing structure back to the inward-facing conformation [85].

#### 4. The physiological function of P-glycoprotein

PGP is expressed in different regions of the body, including the brain, kidneys, liver, gastrointestinal tract, testis and placenta. PGP is an essential component of the blood brain barrier (BBB) and is expressed on



**Figure 2.** Nucleotide binding domain with the walker regions (ABC). The three regions facilitate ATP binding and hydrolysis, represented in different colours (A: blue, B: green, C: red) enclosed by the black hexagon shape. Overall structure is illustrated using ribbon representation and coloured differently according to the transmembrane (TM) and nucleotide binding domains (NBD).



**Figure 3.** Two PGP transporters in different conformations across the phospholipid bilayer. The inward-facing structure (left) promotes drug binding, while the outward-facing structure (right) facilitates the efflux process. The channel pore for the outward-facing structure is narrow, hence the drug is actively pumped out into the extracellular environment.

the apical surface of capillary endothelial cells, restricting the entry of foreign toxic substances into the brain [101, 102]. Resistance to pilocarpine during seizure induction has been observed in animal models of epilepsy and PGP is thought to be responsible for this resistance [103, 104]. Compared to wild-type mice, PGP knockout mice require a lower dose of pilocarpine for seizure induction [104]. Other studies have also reported PGP knockout mice having a 17- to 83-fold increase in substrate concentration in the brain, whereas the levels in other organs, such as the liver, kidneys and intestines, were only increased by 2- to 3-fold [105]. In humans, a 129% increase in the brain concentration of (R)-[<sup>11</sup>C] verapamil [106] and a 223% increase of [<sup>11</sup>C]N-desmethyl-loperamide [107] were observed when patients were administered 6 mg/kg of tariquidar, a PGP inhibitor. In another recent positron emission tomography (PET) imaging study, administration of tariquidar resulted in a 273% ± 78% increase in the brain concentration of (R)-[<sup>11</sup>C] verapamil [108], further highlighting the protective role of PGP at the BBB.

PGP also has an efflux function in the kidneys, where it is localised on the apical surface of the proximal tubule epithelial cells [109]. Elevated PGP levels in the kidneys in response to endotoxemia [110] and ischemia reperfusion injury [111] have been recorded in several animal studies. In another study, higher levels of *de novo* PGP synthesis were observed in the kidneys upon exposure to the proinflammatory cytokine tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), lipopolysaccharide (endotoxin) or a combination of both TNF- $\alpha$  and lipopolysaccharide [112]. Similar observations have also been made in other *in vivo* lipopolysaccharide exposure studies [110, 113, 114]. Therefore, it has been suggested that PGP protects the kidneys by preventing the accumulation of toxic substances in cases of injury.

Some studies have also suggested that PGP is involved in cholesterol trafficking [115, 116, 117], as well as lipid homeostasis [118, 119]. Cells expressing higher levels of PGP have been noted to undergo enhanced esterification of the plasma membranes [117]. PGP knockout mice have demonstrated higher insulin and glucose levels compared with wild-type mice [119]. These mice also weighed more compared with the wild type and suffered from adipose hypertrophy resulting in hepatosteatosis (fatty liver disease). This hepatosteatosis was marked by an increase in the expression of liver detoxification genes and *de novo* lipid synthesis, as the liver is normally a vital site for lipid homeostasis. It is possible that downregulation of PGP affects the bioavailability of lipids, as they are PGP substrates, which forces the use of other metabolic pathways resulting in obesity. Furthermore, the knockout of PGP can also affect biliary excretion in the liver [120, 121]. In humans, the downregulation of PGP at the BBB has also been linked to higher rates of obesity [122]. Thus, PGP has proven to be essential for a healthy body mass index.

Although PGP is well known for its effect on the efflux of xenobiotics, studies have suggested another intriguing role for PGP in listeriosis [123]. *Listeria monocytogenes* is a Gram-positive bacterium and a common food-borne pathogen that begins its pathogenesis in the gastrointestinal tract in the Peyer's patches [124, 125], intestinal villi [126] and goblet cells [127] via the internalins A and B. During this process, the intestines are exposed not just to the bacteria but also to several surface-attached proteins generated by the bacteria. Both *in vivo* and *in vitro* experiments have shown that the inhibition of PGP facilitates this bacterial invasion, whereas overexpression of PGP results in resistance to this invasion [123]. PGP knockout mice exhibited a higher incidence of infection compared with the wild-type counterparts. Expression of PGP resulted in the efflux of the bacterial proteins from the basolateral surface to the apical surface, whereas the inhibition of PGP resulted in a decline of this flow [123]. Another study later reported that PGP-mediated activation of the S100A8/S100A9 complex was responsible for this resistance to infection via the phosphorylation of Ser552 by  $\beta$ -catenin [128]. PGP was shown to reduce PI3K $\alpha$  phosphorylation by the bacteria, which reduced the likelihood of the bacteria invading the intestinal cells through gap junctions. In listeriosis, the bacteria replicate in the cytoplasm and infect the neighbouring cells without causing cell lysis [129]. Upon sensing this replication in host cells, cytosolic receptors of the innate immune system activate the type I interferon response resulting in the secretion of interferon-beta (IFN- $\beta$ ) [130]. Recent studies have shown that PGP is essential for this innate response [131]. PGP expression elevates the type I interferon response whereas blockade of PGP reduces the expression of IFN- $\beta$  in infected cells. PGP is also crucial for the activation and migration of dendritic cells from the periphery to the lymph nodes [132, 133, 134]. The inhibition of PGP by venlafaxine prevents the differentiation of dendritic cells [135]. This inhibition also diminishes the polarisation and proliferation of T cells, as well as the production of cytokines. In addition to dendritic cells, PGP expression has also been observed in CD4<sup>+</sup> T regulatory cells, T effector cells, CD8<sup>+</sup> cytotoxic T cells and natural killer cells [136, 137, 138, 139]. PGP expression has been shown to play a role in FTY720-mediated T cell migration as PGP can transport sphingosine-1-phosphate [140]. PGP is essential for the normal development of T regulatory cells [141] and also protects T helper cells (Th1 and Th17) from bile acid-driven oxidative stress in the small intestine [142]. Upon viral infection, PGP appears to initiate the production of T effector cells, whereas in a bacterial invasion PGP has a protective function towards T memory cells [139]. It has been reported that PGP-expressing polyclonal CD8<sup>+</sup> T cells exhibited greater effector memory phenotypes compared with naïve cells lacking PGP. Follow-up *in vivo* studies reported that inhibition of PGP resulted in an upregulation of

genes and transcription factors related to autophagy and apoptosis. PGP downregulation also reduced ATP production and increased the generation of MitoSOX, which is an indicator of oxidative stress [143, 144]. Thus, normal-functioning PGP ensures cell survival by suppressing oxidative stress and preserving mitochondrial function in developing T cells [139].

In the testis, PGP has been shown to interact with the tight junction protein zonula occludens 1 (ZO-1), focal adhesion kinase (FAK), junctional adhesion molecule A (JAM-A), claudin-11 and occludin, playing an essential role in the restructuring of the blood-testis barrier (BTB) during spermatogenesis [145, 146]. The seminiferous epithelium of the BTB exist as two separate regions, the apical and the basal chambers [147]. This separation ensures that spermiogenesis can safely take place in the apical region without the immune system creating antibodies against the newly formed cells [147, 148]. When these cells reach stage VIII of their cycle, the entire BTB undergoes a substantial amount of restructuring wherein PGP plays an important role. The knockdown of PGP has been shown to increase the FAK-occludin interactions, resulting in phosphorylation of the serine and threonine residues in occludin, which destabilises the occludin/ZO-1 adhesion complex [146, 149]. This destabilisation reduces the adhesion between the Sertoli cells, therefore, making the BTB more permeable. In immunohistochemistry studies using mice, rats and human testicular sections, PGP has been detected in various somatic testicular cells, such as the Leydig cells, macrophages, peritubular cells and Sertoli cells [150]. In rats, these cells exhibited MDR suggesting a defensive role for PGP in germ-line cells [150].

PGP has also been detected in proliferating cytotrophoblast (CT), syncytiotrophoblast (ST) and extravillous trophoblast (EVT) cells, highlighting a possible role for PGP in placental development [151]. PGP is involved in both the invasion and migration of CT and EVT cells, ensuring a proper connection is formed between the circulation of the mother and the foetus [151, 152]. The knockout of PGP resulted in complete obstruction of the EVT invasion and migration. This obstruction resulted in the formation of abnormal placental tubes that were longer and more complex [151] than normal tubes. Similar results were also observed for primary EVT cells in vasculogenesis and trophoblast syncytialisation. Lower PGP levels have also been reported in severe early-onset pre-eclamptic placentas and preterm placentas with acute chorioamnionitis [151, 153]. Besides aiding in placental development, PGP also plays a pivotal role in the placental barrier, protecting the unborn foetus from maternally transported steroids, toxins and xenobiotics [151, 154, 155]. However, the expression levels of PGP have been known to vary among individual foetuses, even those in the same gestational period [156, 157], thus affecting the level of exposure of the foetuses to toxic substances. Studies in humans and mice have reported that PGP levels were the highest during early gestation to protect the unborn foetus during this developmentally sensitive stage, with hCG- $\beta$  being implicated for this upregulation of PGP in humans [158, 159]. The importance of PGP was further highlighted in a study that exposed pregnant PGP knockout mice to a teratogenic substance. Because these foetuses had no protection against the teratogen, 100% of them were born with cleft palate abnormalities, while the wild-type foetuses exhibited no birth defects from the teratogenic exposure [154]. In addition to the placenta, PGP has also been detected in the foetal brain [160, 161, 162], liver [163, 164, 165, 166], kidneys [164] and small intestine [164, 167]. Although the exact role of PGP in the developing foetal organs has not been clearly elucidated, it is possible that PGP serves a protective role, similar to that in healthy adults.

## 5. The regulation of PGP

Because of its diverse functions in the body, a variety of factors have been reported to regulate the functions and expression levels of PGP. Several animal studies have reported that the neurotransmitter glutamate positively regulated PGP expression and the transport function of PGP in the brain via N-methyl-D-aspartate (NMDA) receptors [168]. Through the

NMDA receptors there is a huge influx of calcium ions into the cell, which activates phospholipase A2 [169]. Phospholipase A2 is a catabolic enzyme that breaks down phospholipids to release arachidonic acid [170]. This increased output of arachidonic acid from the cell is then converted into prostaglandins by cyclooxygenase-2 (COX-2) [171]. Several studies have highlighted a connection between NMDA, COX-2, and PGP signalling [168, 172, 173, 174, 175]. Increased levels of COX-2 have been shown to elevate the levels of PGP expression [176, 177, 178]. Steroid hormones, such as oestradiol, have also been identified as modulators of PGP [179]. Activator protein-1 (AP-1) and nuclear factor kappa B (NF- $\kappa$ B) are other known inducers of PGP [180]. Elevated PGP expression levels were observed when NF- $\kappa$ B was translocated to the nucleus in response to AKT phosphorylation via the JNK pathway [181]. Other MAPK signalling pathways, such as the ERK [182, 183] and p38 pathways, [184, 185] have also been reported to elevate PGP expression levels. Both the PI3K/Akt/mTOR pathway [186, 187, 188] and Wnt/ $\beta$ -catenin signalling pathway [189, 190, 191] have also been shown to upregulate PGP expression. Pro-inflammatory cytokines, such as TNF- $\alpha$ , are also known inducers of PGP [112, 192, 193]. The transforming growth factor-beta 1 (TGF- $\beta$ 1)/Smad signalling pathway has also been recognised as a positive regulator of PGP [194], with studies showing TGF- $\beta$ 1 activity being mediated through both the ALK1 and ALK5 pathways [195]. TGF- $\beta$ 1 activity can also be modulated by glucocorticoids [196], which also possess the ability to regulate PGP expression levels directly [197, 198, 199, 200, 201]. The ABCB1 gene itself can also be modulated by epigenetic mechanisms, such as histone acetylation [194, 202, 203, 204]. The promoter region of PGP is a common binding site for several transcription factors, including the leucine-rich PPR-motif-containing protein [205,206], p53 [207,208,209,210], MYCN [211, 212, 213, 214], SP-1 [215,216] and YB-1 [217,218]. Methylation of the promoter region has also been suggested to play a role in PGP induction [219, 220, 221]. Several studies have also reported that microRNAs (miRs), such as miR-145, miR-27a and miR-331-5p, interact with the 3' untranslated region (UTR) of ABCB1 mRNA, thus reducing PGP expression levels [222, 223]. Other miRs, such as miR-137, can modulate transcription factors, such as YB-1, and thus indirectly decrease PGP expression [224, 225]. miR200c has been observed to downregulate the expression of the JNK2 gene, thus inhibiting JNK2/p-JNK/p-c-Jun/ABCB1 signalling and lowering PGP expression levels [226]. Studies have also identified that small GTPases, such as Ra1A, Rab4 and Rab5, can modulate the trafficking and surface expression of PGP [227, 228].

## 6. PGP alterations in disease

Because a wide variety of factors can modulate the function and expression levels of PGP, a role for PGP in diseases is inevitable. p53 is a protein often called "the guardian of the genome" that possesses potent tumour suppressive functions. p53 regulates cell-cycle arrest, apoptosis, senescence and autophagy in response to cellular stress, DNA damage, oncogene activation or hypoxia [229]. However, mutations in p53 suppress these apoptotic pathways resulting in cancer [230]. Nuclear accumulation of mutant p53 has been shown to elevate PGP expression levels, generating an aggressive MDR phenotype in breast cancer and acute myeloid leukaemia patients, resulting in a poor prognosis [231, 232]. The contribution of mutant p53 to MDR in cancer has been observed in several studies [233, 234]. The chemotherapeutic drug doxorubicin is often used in hepatocellular carcinoma to induce the apoptosis of malignant cells via the p53 pathway [235]. Studies have shown that defective p53 genes can result in doxorubicin resistance [236], whereas overexpression of wild-type p53 genes promoted chemosensitivity [237]. Similarly, studies have reported higher expression levels of PGP in doxorubicin-resistant HepG2 cells [238, 239], while downregulation of PGP resulted in increased sensitivity to the drug [240]. Inhibition of the oncogenic serine/threonine kinase Pim-1 has also been shown to increase doxorubicin sensitivity in PGP-positive cells [241]. This result is hardly surprising as several chemotherapeutic drugs are PGP substrates. In

cancer, Pim-1 protects PGP from ubiquitination and proteasomal degradation and ensures PGP glycosylation and increased cell surface expression, which can lead to MDR. Both the MAPK signalling pathway and the COX-2 gene have been implicated in PGP-mediated MDR in hepatocellular carcinoma [239, 242]. Mutant p53 has also been shown to interact with the ABCB1 promoter in osteosarcoma and colon carcinoma [243]. Overexpression of (chemokine receptor type 4) CXCR4 is another factor known to contribute to higher expression levels of PGP in cancer [244, 245, 246, 247]. CXCR4 is responsible for the recruitment and activation of immune cells, as well as the production of various cytokines [11], which in the case of cancer leads to the migration of inflammatory and metastatic cells. Studies have reported a poor prognosis for PGP-positive paediatric ependymoma patients as these individuals exhibit increased drug resistance, local invasion and tumour recurrence [248]. A study on oesophageal carcinoma cells revealed that downregulation of miR-27a resulted in lower levels of Bcl-2, ABCB1 transcription and PGP [249]. Elevated PGP levels and doxorubicin resistance were also observed in breast cancer cells when miR-298 was downregulated [18]. Targeted inhibitors of PGP that interact with the NBD, instead of the substrate binding sites, have been shown to reverse MDR and increase tumour cell senescence through chemotherapy [6], further highlighting the role of PGP in cancer.

Elevated expression levels of PGP have also been reported in cases of peripheral inflammatory pain with increased PGP trafficking from nuclear stores to the endothelial cell luminal membrane [250]. Several opioid analgesics, such as morphine, are also PGP substrates making it difficult to design a pain management regimen [44]. Therefore, several recent studies have attempted to improve drug delivery by administering milder analgesics in conjunction with morphine [251, 252]. The use of acetaminophen results in the activation of the constitutive androstane receptor, which elevates PGP levels at the BBB [251]. Comparatively, pre-treatment with the non-steroidal anti-inflammatory drug (NSAID) diclofenac reduces morphine uptake, but the concurrent administration of diclofenac with morphine improves drug uptake even though the PGP expression levels are high, suggesting a specific drug-drug interaction [252]. Morphine-tolerant rats have been reported to have a 2-fold increase in PGP trafficking from the nuclear stores to the endothelial cell luminal membrane [44]. Studies have also shown that peripheral inflammatory pain can result in specific structural changes in PGP, which in turn could affect the role of PGP in drug delivery [12].

High expression levels of PGP have also been observed in post-operative peritoneal adhesions via the activation of the TGF- $\beta$ 1/Smad signalling pathway and histone H3 acetylation [194]. Elevated levels of PGP have been shown to amplify phosphorylation of the chloride channel-3 to modulate the cell volume [194, 253]. This modulation increased the proliferation and migration of fibroblasts through volume-activated chloride currents resulting in the formation of peritoneal adhesions. TNF- $\alpha$  has been reported to activate B lymphocytes and upregulate the surface expression of PGP in autoimmune disorders, such as RA [254]. PGP levels have been detected to be particularly high in CXCR4-overexpressing B lymphocytes [11]. Thus, disease modifying antirheumatic drugs are pumped out of the system, mediating MDR in patients with active RA. Studies have also reported higher levels of PGP-expressing CD4<sup>+</sup> cells in the peripheral blood and renal tissue of lupus nephritis patients suffering from MDR [10]. PGP knockout animals have shown impaired dendrite cell maturation and decreased T cell stimulation, and exhibited symptoms of experimental autoimmune encephalomyelitis, suggesting a possible role of PGP in multiple sclerosis [255].

Various animal models and human studies have highlighted the role of the overexpression of PGP at the BBB in pharmacoresistant epilepsy [33, 256, 257, 258, 259, 260]. It has been suggested that recurrent seizure activity results in additional extracellular glutamate release that leads to the activation of NMDA receptors and elevated COX-2 levels [168]. Elevated COX-2 levels are known to increase PGP expression levels, resulting in the large-scale efflux of anti-epileptic drugs [176].

Elevated PGP levels have also been identified in neurodegenerative disorders, such as ALS [9, 261, 262]. In some forms of ALS, sporadic astrocytes that modulate PGP expression through NMDA receptors have been shown to be present [262].

Lower expression levels of PGP because of genetic variants of the ABCB1 gene have been associated with an increased likelihood of Parkinson's disease (PD) [263]. PET scans and biochemical studies on PD patients have indicated that there is a significant reduction in PGP activity in these patients [264, 265]. Compared with controls, there was an increased uptake of <sup>11</sup>C-verapamil into the brain suggesting an attenuation of the PGP efflux function. Similarly, PGP levels have also been shown to be downregulated in Alzheimer's disease (AD) [266, 267]. In healthy individuals, PGP is responsible for the removal of the amyloid- $\beta$  protein (A $\beta$ ) from the brain [266, 268, 269, 270, 271, 272, 273, 274]. A $\beta$ , which is a major hallmark of AD, is also a PGP substrate [275], and the downregulation of PGP has been shown to elevate the accumulation of A $\beta$  in the brain [267, 270, 276, 277].

## 7. Current challenges and future possibilities for drug development

Over the years, several attempts have been made to include PGP inhibitors as part of the drug regime for patients with MDR diseases, such as cancer. Verapamil, a voltage-dependent L-type calcium channel blocker, was one of the earliest drugs to be tested as a PGP inhibitor [278]. Patients with small cell lung cancer in a phase II trial were administered 480 mg of verapamil along with the usual chemotherapy drugs (cyclophosphamide, doxorubicin, vincristine, and etoposide; CAVE) in an attempt to improve drug uptake and efficiency [278, 279]. Although the verapamil dosage used was near the maximum tolerated dose, no positive effects were observed in the patients [278, 279]. Furthermore, the concentration of verapamil in the blood was significantly lower than the required efficacious dose reported in preclinical studies [278, 279]. Patients also reportedly suffered from hypotension [279]. Another famous PGP inhibitor tested was dexverapamil, the R-form of verapamil, which had lesser potency as a PGP inhibitor but also fewer risks of cardiotoxicity. When used in a phase II trial alongside anthracyclines to battle chemoresistance, partial response was observed in 2 of the 21 patients, with only a 19% disease control rate [278]. However, the toxicity levels were more tolerable than its predecessor [278]. More recently, scientists have developed a new generation of PGP inhibitors, such as tariquidar and zosuquidar, with the intent of optimizing potency (10–100 nM) and enhancing specificity to PGP [280]. However, studies have reported contradicting results on the role of tariquidar as a PGP inhibitor [280]. Such conflicting evidence prevent drugs from proceeding to the later phases of clinical trials and very few receive FDA approval due to reports of poor efficiency and high toxicity levels. The current known computational methods of predicting drug-protein interactions include quantitative structure activity relationship [281], classification models [282], and molecular docking [283], to name a few. Our current knowledge of the PGP structure, will tremendously aid this process as several of these methods rely on the accuracy of the three-dimensional structure of the protein. Additionally, learning the pivotal role that PGP plays in physiological function is of great importance because if we were to potentially design a strong inhibitor in the future that could negate the efflux function of PGP, there is a possibility of this drug interfering with the usual protective function of PGP which could prove detrimental to the organs where it is expressed in healthy levels. Moving forward, it might also be beneficial to design drugs based on properties other than the inhibition of PGP. For instance, PGP is known to interact with hydrophobic compounds. Therefore, chemically modifying the drug to possess a polar moiety or attaching a polar side chain to it could potentially reduce the efflux function of PGP. Alternatively, rather than designing new PGP inhibitors, it might be more feasible to repurpose already known drugs that have previously been approved by the FDA for the treatment of other illnesses. This method is highly advantageous as it

avoids the numerous and time-consuming preclinical toxicity tests, thus lowering drug manufacturing costs in the process. In addition, scientists could also try targeting sites that are involved in the opening and closing mechanisms of the pump. If the PGP structure is restricted to either the inward- or outward-facing conformation it could potentially reduce the frequency of drugs being pumped out of the system. Besides traditional drug development there are also newer methods such as CRISPR/Cas9-based genome editing that have shown some promise in reversing MDR. As reported by Yang and colleagues (2016), the employment of CRISPR/Cas9 to knockout ABCB1 significantly improved sensitivity to rhodamine 123 and doxorubicin with a marked increase in the intracellular accumulation of these drugs in cancer cells [284]. Another interesting method to combat PGP-induced MDR could be the incorporation of microRNAs (miRNAs). miRNAs possess the ability to regulate gene expression through RNA interference or gene silencing. This means they could inhibit the translation of ABCB1 mRNA into PGP proteins, thus reducing its expression levels. A good example of this is the utilization of miR-298 to reverse MDR in refractory epilepsy [285]. This miRNA was shown to downregulate PGP expression levels while also improving internal accumulation of anti-epileptic drugs [285]. However, these techniques are still in their early stages of development and will require further studies to elucidate their underlying mechanisms.

## 8. Conclusion

Because of the various roles PGP plays in both health and disease, it is difficult to combat PGP-induced MDR. In healthy individuals, PGP plays a crucial role in the development of an immune response, cholesterol metabolism, and the differentiation and migration of various cells, while also serving a protective function in several organ systems. PGP is an inherent barrier that protects the body from xenobiotics and other toxic substances. Thus, any changes in the expression levels of PGP can be detrimental. In the case of pregnant women, knockdown of PGP can also be dangerous for the developing foetus. Therefore, with regard to MDR, simply inhibiting PGP function is not an option, as this might reduce drug resistance, but it may also give rise to other health complications. Designing suitable therapeutic drugs to bypass the PGP transporter is a viable option, provided that these drugs are not PGP substrates. The administration of a suitable competitive inhibitor concurrently with the therapeutic drug is another possible option. In theory, this method would ensure that there is more than one substrate competing to bind with PGP, therefore perhaps decreasing the likelihood of the therapeutic drug being pumped out of the cell. Unlike a non-competitive inhibitor, a competitive inhibitor might not completely shut down PGP function, thus possibly allowing maintenance of the regular physiological functions of PGP. Recent studies have highlighted new aspects of the PGP structure and this knowledge could be exploited to design drugs that target other regions of the PGP complex outside of the substrate binding regions.

## Declarations

### Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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No data was used for the research described in the article.

## Declaration of interest's statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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