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Multidrug resistance in the protozoan parasite *Entamoeba histolytica*

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Abstract

In this review we discuss the mechanisms and molecules involved in the multidrug resistance (MDR) of the protozoan parasite *Entamoeba histolytica.* Drug resistant mutants exhibited the main characteristics presented by the MDR mammalian cells. They showed cross-resistance to several unrelated drugs that is reverted by calcium channel blockers. MDR phenotype in *E. histolytica* is regulated at a transcriptional level by the *EhPgp1* gene, which is constitutively expressed and by the *EhPgp5* gene, whose expression is induced in the presence of the drug. Transcription factors participate in the expression regulation of these genes. After over transcription, the *EhPgp* genes are amplified, cooperating to produce the MDR phenotype. Post-transcriptional mechanisms such as mRNA stability seem to be involved in this phenomenon. As for other *mdr* gene products, the EhPGP5 protein functions as a chloride current inductor or as a regulator of cellular regulatory volume decrease.

Keywords: Multidrug resistance; Protozoan parasite; *Entamoeba histolytica*

1. Introduction

Protozoa are responsible for some of the most important and prevalent diseases in humans, threatening the lives of nearly 1250 million humans around the world. There are no effective vaccines against protozoan parasites, thus prevention and drug administration are the weapons used to fight them. A serious preoccupation is related to increased widespread resistance to many anti-parasitic drugs, as has occurred with chloroquine-resistance in malaria and metronidazole-resistance in anaerobic protozoa [1]. The development of new anti-parasitic drugs is proceeding slowly and vaccines are not yet in sight, therefore the raising of drug resistance is seriously menacing the control of protozoan diseases. Upon this panorama, the study of the factors involved in drug resistance mechanisms developed by parasites has today the highest priority.

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Entamoeba histolytica is the protozoan responsible for human amoebiasis. It affects 500 million people, causes 50 million clinical cases of dysentery or liver abscesses, and kills 100 000 persons/year around the world [2]. Amoebiasis is controlled mainly by treatment with metronidazole derivatives and emetine [3]. Apparently before, drug resistance was uncommon in amoebiasis [4], however, drug-resistant strains were generated in the laboratory [5,6].

Differences in drug susceptibilities have been reported [7] and case reports of failed drug treatments have been augmented [8], suggesting that drug resistance can occur in parasites inside the human host. The emergence of a drug resistance phenomenon in *E. histolytica* could limit chemotherapy, representing a major challenge to disease treatment. In this paper, we reviewed some of the mechanisms and molecules involved in *E. histolytica* drug resistance.

2. Physiology of multidrug resistance in *E. histolytica*

To study the physiology and molecular basis of drug resistance in *E. histolytica,* we generated multidrug resistant trophozoites using the alkylating agent ethylmethanosulphonate [6]. Drug resistant trophozoites were selected after mutagenesis by their ability to grow in emetine. The trophozoites of the wild-type clone A are sensitive to 8 μM emetine, whereas the mutant trophozoites of clone C2 grew well in 90 μM emetine and presented cross-resistance to colchicine, diloxanide and iodoquinol [6,9]. Later, trophozoites of clone C2 were step selected to obtain trophozoites resistant to 225 μM of emetine [C2(225)]. Drug uptake experiments using radiolabeled ^{3}[H]-emetine and ^{3}[H]-colchicine, showed that the multidrug resistant trophozoites accumulated less drug concentration than the sensitive cells. Their resistance was reverted by verapamil, a calcium channel blocker [9,10], resembling the multidrug resistance (MDR) phenotype described in mammalian tumoral cell lines [11,12] and in other parasites [13–15].

MDR phenotype involves the overexpression of a 170 kDa membrane P-glycoprotein (Pgp), which acts as an ATP-dependent efflux molecular pump, decreasing the intracellular drug concentration [12]. Pgps are conformed by two homologous halves, each one containing an ATP binding cassette (ABC) linked to six-transmembrane α helices [16,17]. Recently, the first crystal structure obtained for an ABC transporter that presents high sequence homology with Pgps was reported in bacteria [18]. The structure confirmed that this protein family possesses six helix bundles that form an inverted V-shaped intramembrane transport chamber, which carries the substrate binding sites. The intracellular region contains the highly conserved nucleotide-binding domains, which couple ATP hydrolysis to perform the substrate translocation [18].

3. *EhPgp* genes in *E. histolytica*

The presence of a typical MDR phenotype mediated by the overexpression of Pgp was unequivocally confirmed by the cloning of four *mdr*-like genes in *E. histolytica* (*EhPgp1*, *EhPgp2, EhPgp5, EhPgp6* genes) (Fig. 1). This is one of the largest *mdr* gene families found in an organism. *EhPgp* genes products have 38–41% identity to the mammalian Pgps and only 22–27% and 11–18% to those of *Plasmodium* and *Leishmania*, respectively [19,20]. Additionally, in silico analysis showed that the hydrophathy plots of the predicted EhPGP amino acid sequences are nearly superimposable onto the human Pgp protein, suggesting that EhPGPs are also conformed by two identical halves, each one containing six putative transmembrane domains, followed by the highly conserved ABC transporters family signature [19].

Molecular characterization of *EhPgp* genes and their products, in sensitive and resistant trophozoites suggest that the expression of the MDR phenotype may be regulated by: (i) the presence of certain factors and sequences promoting differential *EhPgp* genes expression; (ii) post-transcriptional events involved in the *EhPgp* mRNA stabilization; (iii) the amplification of the *EhPgp* genes; and (iv) the particular physiology that each one of the EhPGPs could present in different cell conditions.

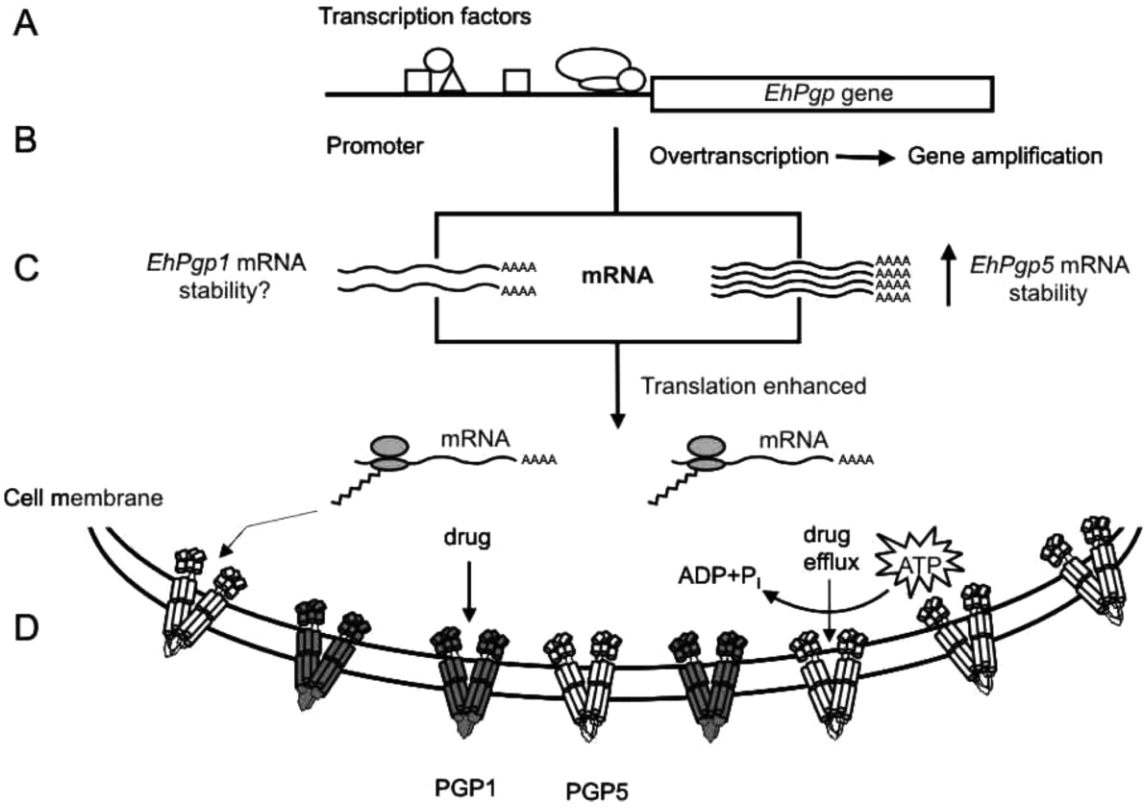

Fig. 1. Diagram of the putative mechanisms involved in the expression of the MDR phenotype in *E. histolytica*. (a) The *EhPgp1* gene is constitutively expressed in drug resistant trophozoites, while the *EhPgp5* gene is overexpressed under drug pressure. The differential expression of both genes in drug sensitive (clone A) and resistant trophozoites (clone C2) is due to the interaction of transcription factors with DNA binding sequences of gene promoters. (b) The stability of the *EhPgp1* mRNA is unknown yet. In trophozoites grown at high drug concentration the *EhPgp1* and *EhPgp5* genes are amplified. (c) The *EhPgp5* mRNA is more stable in trophozoites of clone C2(225) permitting the synthesis of a higher amount of EhPGP5 protein by the translation machinery. (d) The EhPGPs drive the drug to the plasma membrane and then pump it out, or change the cell permeability modulating chloride channels, avoiding the entrance of the drug.

4. Transcriptional regulation of the *EhPgp* genes

In drug resistant mutants the *EhPgp1* and *EhPgp6* genes are constitutively expressed, while the *EhPgp5* gene is transcribed only in the presence of the drug and the *EhPgp2* transcript has not been detected yet [20,21]. In drug sensitive cells, the *EhPgp1* mRNA was detectable only by RT-PCR and primer extension experiments, but the *EhPgp5* gene has not been detected by different sensitive techniques, thus it is probably that *EhPgp5* gene is not expressed in drug sensitive trophozoites. Structural and molecular characterization of promoters indicated that transcriptional regulation is important for the differential *EhPgp* genes expression (Fig. 1)

The *EhPgp1* and *EhPgp5* gene core promoters are composed by approximately 300 bp upstream the transcription initiation site. Their sequences are almost identical in drug sensitive and drug resistant mutant trophozoites. However, gel shift assays evidenced that nuclear proteins from trophozoites of clones A or C2 generate differential complexes with DNA binding consensus sequences present in the *EhPgp1* and *EhPgp5* promoters [22,23], suggesting that the concerted interplay of transcription

proteins are involved in the up and down regulation of the genes. Transfection experiments gave further evidence on the involvement of transcriptional events in the *E. histolytica* MDR phenotype, and the multiple protein–DNA interactions needed for the *EhPgp1* and *EhPgp5* genes expression.

4.1. The EhPgp1 gene expression regulation

The *EhPgp1* core promoter does not have a typical TATA box but has two putative Inr sequences, probably involved in recruiting the transcription preinitiation complex. One Inr site is located at −18 bp (GAACTA) and contains the CE2 motif (GAAC) [22,24], and the other is at −67 bp (TTAGATT), close to a C/EBP consensus binding sequence (−54 to −43 bp). This promoter region (−1 to −74 bp) forms a DNA–protein complex, detectable exclusively with nuclear extracts (NE) from drug resistant trophozoites, which is competed by oligonucleotides carrying the C/EBP consensus binding sequence [22]. At −259 to −168 bp the promoter has HOX, C/EBP, POU and GATA-1 consensus binding sequences, which form a DNA–multiprotein complex, also exclusively with NE from trophozoites of clone C2 [22].

Plasmids carrying the *EhPgp1* gene promoter with mutations in C/EBPs (−54 to −43 bp and −198 to −186 bp), and GATA-1 (−228 to −223 bp) sites did not drive CAT gene expression in transfected trophozoites of clone C2. These results indicated that both C/EBP and GATA−1 DNA binding sites and proteins that bind to these sites play a relevant role in promoting *EhPgp1* gene transcription. Antibodies against the human C/EBP proteins recognize a protein in the nucleus and EhkO organelle ([25], Marchat et al., unpublished results). EhkO is a mithocondrion-like organelle discovered first in the cytoplasm of the trophozoites by its DNA content [25]. We hypothesize that the protein interacting with the *EhPgp1* promoter site at −54 bp, in the C/EBP site, stabilizes the transcription preinitiation complex in the proper site. At the same time, other protein binding at −198 bp, in the other C/EBP site participates in the formation of the multiprotein complex and probably provokes the DNA folding to allow the interaction of both proteins, and the other factors forming the multiprotein DNA complex, favoring the *EhPgp1* gene expression.

4.2. The EhPgp5 gene expression regulation

The *EhPgp5* gene promoter contains a TATA box-like motif at −31 bp of the transcription initiation site [23]. The *EhPgp5* gene is transcribed in response to emetine in a dose-dependent manner, suggesting that its expression could be regulated by transcription factors, where synthesis may be induced by the presence of the drug. Furthermore, the *EhPgp5* mRNA 5′ end maps three bases upstream of the start codon in clone C2(225) trophozoites, but a truncated mRNA was detected in the absence of emetine, suggesting that gene expression could also be associated with the accurate selection of the transcription initiation site [23]. CAT gene expression is driven by the *EhPgp5* gene promoter only in drug resistant transfected trophozoites, but its activity is very low in cells cultured without emetine. The promoter contains several putative consensus sequences for transcription factors including AP-1, HOX, C/EBP, OCT-1, OCT-2 and MYC-like proteins. Putative HOX and AP1-like factors form specific DNA–protein complexes with NE from clone C2(225) trophozoites, which were undetected in drug sensitive trophozoites. Moreover, according to gel shift assays, unidentified factors expressed in the drug sensitive trophozoites seem to interfere with the binding of the TATA-binding protein, producing a loss of promoter activity in clone A or inducing the selection of a wrong initiation start site in the trophozoites of clone C2 grown without emetine [23]. Thus, in the trophozoites of clone C2 (225) may exist transcription factors involved in the induction of *EhPgp5* gene expression, which seems to be related to the presence of emetine in the medium.

5. EhPgp genes amplification

In transformed mammalian cells, *mdr* genes are amplified at a 100 or 1000-fold excess of drug concentration, after cells have reached high *mdr* mRNA levels [26]. This phenomenon has also

Table 1
Differential *EhPgp* mRNA expression and gene amplification in drug sensitive and drug resistant trophozoites of *E. histolytica*

Clone	Emetine susceptibility (μM)	Genes (mRNA expression/gene amplification)			
		EhPgp1	*EhPgp2*	*EhPgp5*	*EhPgp6*
A	8	−/−[a]	−/−	−/−	−/nd
C2	90	+++/−[b]	−/−	+/−	++/nd[c,d]
C2 (225)	225	+++/++	−/−	++++/++	nd/nd

[a] −, not *EhPgp* mRNA expression or not *EhPgp* gene amplification.
[b] +, *EhPgp* mRNA expression or *EhPgp* gene amplification.
[c] nd, Not determined.
[d] *EhPgp6* mRNA levels were determined for clone C2 grown with 24 μM emetine.

been observed in parasites [13,14]. In *E. histolytica*, dot blot experiments using specific *EhPgp1* or *EhPgp5* gene probes, evidenced that the trophozoites of clone C2(225) also presented *EhPgp* gene amplification [27]. As in *Leishmania*, where *mdr* genes are amplified in the extrachromosomal elements called H and V circles [28], the *EhPgp* genes were located in the cytoplasm, probably in the EhkO organelles, because the probes gave positive reaction in the cytoplasm, and the cytoplasmic DNA of *E. histolytica* trophozoites is contained in the EhkO organelles [25]. In contrast, in the trophozoites of clone A, *EhPgp* genes appear in a lower amount and only in the nucleus. *EhPgp* gene copies coexisting in the cytoplasm and in the nucleus could be selectively amplified in the trophozoites by unknown mechanisms. Surprisingly, *EhPgp* gene amplification was observed only in some trophozoites of clone C2(225). It is possible that to be induced, gene amplification requires a high drug concentration, and trophozoites in culture probably take different amount of drug. Thus, *EhPgp* gene amplification could also participate in the modulation of the MDR phenotype (Table 1, Fig. 1).

6. Post-transcriptional regulation and role of mRNA stability

Transcriptional regulation plays an important role in the *EhPgp* gene expression, however, the relevance of post-transcriptional events cannot be discarded (Fig. 1). In rat, overexpression of the *Pgp1*, *Pgp2* and *Pgp3* genes seems to be also controlled by post-transcriptional mechanisms, involving mRNA stability [29,30]. Our recent studies on the *EhPgp* mRNAs half-life showed that *EhPgp5* mRNA stability is increased in drug resistant trophozoites (López et al., unpublished results). A preliminary analysis of the 3′UTR *EhPgp5* mRNA downstream sequences evidenced that they are extremely AU-rich (85%) and contain several consensus sequences for RNA-binding proteins, which could participate in mRNA stability [31]. However, mRNA turnover mechanisms are almost unknown in *E. histolytica*, and many studies are needed before accurately establishing the effect of *EhPgp* mRNAs stability on the MDR phenotype.

7. EhPgp protein functions

In mammalian cells, the Pgps have been involved in stress response, in removing toxic substances from the cell, cytokines transport, lipid translocation, apoptosis, mechanisms for regulatory volume decrease (RVD), and importantly, as regulators of the volume-activated chloride channels [32]. Transfection of the human *MDR1* cDNA into mammalian cells produces a swelling activated chloride conductance [33]. However, Pgps cannot simultaneously function as pumps and chloride conductance activators [34,35]. Reversible phosphorylation may be the mechanism defining one of the two functions carried out by Pgp [34].

The study of the functions performed by the EhPGP5 protein was recently initiated by transfection experiments of the genes into sensitive tro-

phozoites and by microinjection of its mRNA in *Xenopus laevis* oocytes [36]. Intriguingly, trophozoites transfected with the *EhPgp5* gene slightly increased their drug resistance. Interestingly, transfected trophozoites became globular and augmented their volume, suggesting alterations in their membrane permeability, possibly related to RVD mechanisms [36], as it occurs in other organisms [37].

X. laevis oocytes microinjected with the *EhPgp5* mRNA, expressed the EhPGP5 protein in their membranes and exhibited chloride ion currents, which were not present in the control oocytes and were abolished by the co-microinjection of the antisense *EhPgp5AS* transcript [36]. Exogenous current was outward during depolarizing pulses. The current amplitude decreased by the reduction of the chloride concentration in the medium and was inhibited by chloride channel blockers, but it was poorly modified by verapamil, suggesting that this calcium channel blocker does not act directly on the EhPGP5 protein. Current was not modified by changes in osmolarity of the medium or by hyposmotic shock, as happened with Pgps of other MDR cells [37]. EhPGP5 protein could function as a chloride current inductor or as a co-adjuvant factor to avoid drug accumulation in the cell, making more complex the panorama of the MDR phenotype in *E. histolytica*.

8. Conclusions

Taken all together, these data indicate that transcriptional regulation of *EhPgp1* and *EhPgp5* genes is strongly involved in a cooperative mechanism that result in the MDR phenotype present in the multidrug resistant trophozoites of clone C2 (Fig. 1). However, several transcription factors participating in the up- and down regulation of the genes remain to be identified. The participation of *EhPgp6* and *EhPgp2* genes has not been studied yet. Gene amplification and mRNA stability increase occur after overtranscription of the genes and in the presence of high drug concentrations, but factors regulating these mechanisms have not yet been studied in *E. histolytica*. As *mdr* genes in other organisms, the *EhPgp* gene products present more than one function. The EhPGP5 may act as a drug pump, it also functions as a chloride ion current inductor and could be involved in the RVD mechanism, whereas the function of other EhPGP proteins remain to be investigated.

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