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QUINOLINE-CONTAINING ANTIMALARIALS—MODE OF ACTION, DRUG RESISTANCE AND ITS REVERSAL

AN UPDATE WITH UNRESOLVED PUZZLES

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Abstract—Malaria constitutes one of the major health threats in the tropical and sub-tropical areas of the world. Yet, few advances were made in recent years in revealing the mode of action of the common and most economically affordable antimalarial drugs, the schizontocidal 4-aminoquinolines. Data presented indubitably repudiate the previous notions that these drugs act by either halting the feeding of the parasite on its host erythrocyte cytosol or repressing nucleic acid synthesis due to intercalation into the parasite's DNA. A novel target for drugs is outlined, i.e. they are shown to inhibit *in vitro* the release of iron from acidified host cell cytosol, consisting mostly of hemoglobin, a process that could provide this trace element to the parasite. Resistance to quinoline-containing drugs is the principal reason for the present resurgence of malaria. Drug-resistant parasites accumulate less of these weak base-like drugs in the acidic digestive vacuoles. A kinetic model is presented, indicating that diminishing drug accumulation is due to decreased vacuolar proton pump activity and is not a result of a putative multidrug resistance (MDR) efflux pump. Findings to date on the molecular biology of parasite *mdr* genes are reviewed. These indicate no correlation between gene expression or mutations and phenotypic drug resistance. Reversal of parasite drug resistance by relevant compounds in MDR cancer cells seems to involve mechanism(s) different from the inhibition of the MDR pump in cancer cells.

It is rather surprising that given the global impact of the health and socio-economic problems caused by malaria, and the protracted use of quinoline-containing antimalarial drugs (QCDs†) our knowledge of their mode of action is so inadequate. One wonders whether this deplorable situation is due to the complexity of the subject matter, the pleiotropic nature of their potential pharmacological effects or simply to lack of scientific interest, and/or the absence of sufficient funding. Whatever may be the real reason, one is obliged to assess periodically the state of the art not only to appraise the whence and whither of our investigational efforts but also to attract more inquisitive minds to join in the endeavor to unravel the mechanism(s) of the specific antimalarial action of QCDs which, despite their dwindling efficacy (due to the evolution of drug resistance), are still the most widely used and easily accessible drugs. Several of the topics that will be dealt with in this short essay have been discussed extensively in recent reviews [1–6] and we shall confine our deliberations to recent findings.

QCDs are acidotropic agents

QCDs are directed against the blood phase that causes the pathological consequences of malarial infection. As amphiphilic weak bases, they enter rapidly into the malaria-infected erythrocyte. Being driven by a pH gradient [7], they accumulate inside the acidic compartment of the infected cell that has been identified as the digestive vacuole(s) of the parasite [8]. They cross membranes as a free base and, once they become protonated, their permeability decreases by orders of magnitude [9]. Hence, the extent of their accumulation can be correlated directly to the pH gradient between the acidic compartment and the extracellular medium. The rapid protonation of the accumulated free base causes a temporary alkalinization of the acidic compartment that is counteracted by the vacuolar proton pump [10]. While the first phase of drug entry ($T_{1/2}$ of a few seconds) is rate-limited by the concentration and the permeability coefficient of the free base, the second phase ($T_{1/2}$ or *ca.* 30 min) is determined by the capacity of the H^+ pump. Concurrently, metabolic poisoning [11, 12] and abrogation of the pH gradient [7, 13] quell drug accumulation. Although it had been proposed that drug accumulation results in a rise in vacuolar pH with consequent inhibition of vacuolar enzymes [14], it was later shown that, at pharmacological concentrations of QCDs, the pH is only insignificantly altered [15]. When drug concentration however is sufficiently high (i.e. above pharmacological levels for antimalarial action), the pH of the drug-accumulating compartment increases, since the H^+ pumping capacity is outpaced and/or there is an efflux of protonated drug. This increase in pH is evidently the cause of the toxicity of QCDs to somatic cells [16].

Biochemical manifestations of drug action

Since the diprotic chloroquine can reach millimolar

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† Abbreviations: QCDs, quinoline-containing antimalarial drugs; MDR, multidrug resistance.

Table 1. Effect of QCDs on acid phospholipase A_2 activity in malaria-infected cells

Drug	IC_{50} (mM)	$[C]_0$ (M)
Quinine	0.1	3.2×10^{-7}
Mefloquine	0.3	9.5×10^{-7}
Chloroquine	5.0	5.0×10^{-8}
Amodiaquine	2.0	2.0×10^{-8}

Trophozoite-infected cells metabolically labeled with [^{3}H]oleic acid, were separated from culture by gelatin flotation and lysed in 1% acetic acid. After two washes in phosphate buffered saline, cells were suspended in acetate buffer (pH 4.5), sonicated briefly and incubated at 37°. The hydrolysis of phospholipids as affected by various QCDs was monitored and the IC_{50} value for each drug calculated. These values were then used to calculate the extracellular concentration of each drug needed to achieve a vacuolar concentration equal to the respective IC_{50}. The pH gradient used for the calculations was taken as 2.5 pH units, i.e. vacuolar pH = 4.9.

and the monoprotic quinine and mefloquine tenth millimolar concentrations in the parasite's acidic (pH 4.5–5) digestive vacuole [10], it was not surprising to discover ultrastructural alterations in this organelle and biochemical evidence for the impairment of its function under the influence of QCDs. Undigested endocytic vesicles containing host cell cytosol accumulate under the effect of chloroquine [17] and a long exposure to mefloquine and quinine results in the disappearance of pigment particles and the appearance of myelin-like membrane whorls [18]. The latter effect could result from the inhibition of acid phospholipase A_2 activity (Table 1; Waldman and Ginsburg, unpublished observations) that could also account in part for the demonstrable inhibition of digestion [19]. While inhibition of digestion is immediate, only after a longer exposure to chloroquine are glucose utilization and the synthesis of nucleic acids suppressed [20].

Complexes of QCDs with ferriprotoporphyrin IX *as cytotoxic agents*

Complexes of QCDs and ferriprotoporphyrin IX [21, 22] that can form in the vacuole might also contribute to the inhibition of digestion through their effect on the parasite's acid cysteine proteinase [23]. These complexes have been implicated before in the mechanism of drug accumulation [24] but the kinetics of the process and the failure to demonstrate their presence in the necessary stoichiometric amounts are incompatible with this allusion. Since these complexes can lyse membranes [25], they have been proposed as the causative cytotoxic agent but the integrity of the vacuolar membrane in QCD-treated cells seems both by morphological [17, 18] and biochemical criteria [26] to remain intact, in line with the recent demonstration that the lytic effect is prevented by the presence of serum proteins [27]. This does not mean to say that QCD–ferriprotoporphyrin IX complexes are not involved at all in antimalarial action. In fact, since they can be formed only in malaria-infected red cells, they are good candidates for accounting for the specific antimalarial effect. Of relevance also is the fact that they can cross membranes [28] and reach targets outside the digestive vacuole.

QCDs as DNA intercalators

The proposal that QCDs may act by intercalating into the parasite's DNA and thereby inhibit DNA and RNA synthesis was very popular in the 1950s and 1960s. Indeed, chloroquine and quinine, but not mefloquine, bind to DNA [18, 29] and a structure–activity relationship has been found between the binding of chloroquine analogs and their antimalarial activity [30]. This hypothesis for the mode of action of QCDs has been revived recently [31], although it does not solve the problems of concentration and specificity. The k_d of chloroquine binding to DNA increases with ionic strength and reaches 2.6 mM when the assay system is brought to physiological levels of salt concentration. These are some four orders of magnitude above pharmacological levels. Hence, although high concentrations of chloroquine may inhibit the synthesis of nucleic acids, thus explaining the effect of chloroquine on various eukaryotes [32], this mechanism is neither compatible with the pharmacology nor with the specificity of the antimalarial action of chloroquine.

A no less serious flaw in this hypothesis is the fact that chloroquine has a higher affinity to poly(dG-dC) nucleotides than to other sequences [33]. The DNA of malarial parasites is notoriously rich in A and T compared to the DNA of its host [34, 35]. Since it is hard to foresee how the simple structure of chloroquine could reorganize a specific nucleotide sequence that may be present exclusively in the parasite's DNA, one would expect this drug to be more toxic against organisms whose DNA is GC-rich, at concentrations where it acts as an intercalator. We have recently addressed this issue using compounds that are known to bind specifically to the minor grove of either AT- or GC-rich sequences, and consequently inhibit DNA and RNA synthesis [36]. As expected, we found that compounds having a high affinity for AT sequences were much more toxic to parasites than to mammalian cells. Those binding preferably to GC-rich sequences did not display such selectivity and in some cases were actually more toxic to mammalian cells than to parasites (Ginsburg *et al.*, unpublished observations). It seems that these results furnish another strong argument against the intercalation hypothesis in the case of chloroquine.

The antimalarial action of QCDs is rapid and irreversible

While the impedance of digestive processes by QCDs is indisputable, it is questionable whether it is their ultimate pharmacological target. This enigma is best exemplified by the reversible nature of the inhibition of parasite growth by the protease inhibitor leupeptin [37], while QCDs exert an irreversible effect the extent of which is maximal after 2–3 hr of exposure (Fig. 1 and Ref. 20). Furthermore, if the major goal of host cell digestion is to provide amino acids for parasite anabolism, it is rather puzzling that amino acid esters, that permeate rapidly across

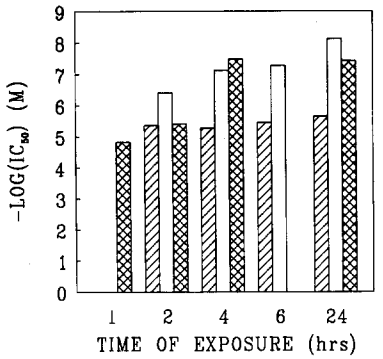

Fig. 1. Irreversibility of the action of QCDs on parasite growth. Parasites at the trophozite stage were exposed to different concentrations of quinine, mefloquine or chloroquine for different lengths of time. The drugs were then washed away and parasites were returned to culture for 24 hr. Parasite viability was determined by their ability to incorporate [^{3}H]hypoxanthine and the IC_{50} values were calculated. Results are expressed in terms of $-\log(IC_{50})$, i.e. the larger the value the higher is the drug sensitivity, vs time of incubation. Chloroquine, cross-hatched bars; quinine, hatched bars; mefloquine, empty bars.

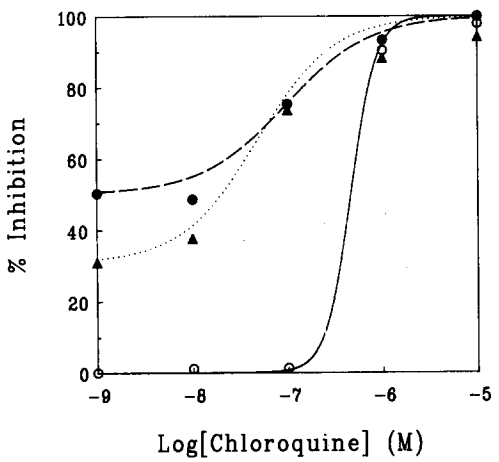

Fig. 2. Combined effect of amino acid ethyl esters and chloroquine on parasite growth. Non-synchronized cultures of *P. falciparum* were cultivated for 48 hr with increasing concentrations of chloroquine in absence (○) or presence of mixtures of ethyl esters (0.1 mM) of essential (▲) (Cys, Glu, Gln, Ile, Met, Pro, Tyr) and non-essential (●) (Ala, Asn, Asp, Gly, His, Leu, Lys, Phe, Ser, Thr, Trp, Val) amino acids. Parasite viability was determined by ability to incorporate [^{3}H]hypoxanthine and the IC_{50} values were calculated to be 4.63×10^{-7} for chloroquine alone, 9.64×10^{-8} for chloroquine + essential and for chloroquine + non essential 4.86×10^{-8} M.

membranes and are hydrolysed intracellularly, are unable to alleviate the inhibition of parasite growth by chloroquine (Fig. 2). In fact, some of these esters synergize chloroquine inhibition, a phenomenon that could hint to the actual mode of action of chloroquine (Krugliak and Ginsburg, in preparation). This result indicates that the amino acid starvation caused by QCDs does not account for their cytotoxic effect on the parasite.

Iron supply—a new target for QCDs

We have discovered recently a new target for QCD action (Gabay and Ginsburg, in preparation). When a lysate of red blood cells is brought to acid pH (pH 4–5), the specific light absorption of hemoglobin declines spontaneously at a rate that decreases with increasing pH. This decline cannot be due to the oxidation of hemoglobin to methemoglobin and clearly indicates changes in the chemical nature of ferriprotoporphyrin IX, the prosthetic moiety of hemoglobin. The rate of hemoglobin degradation increases in the presence of proteinase K or cathepsin D, a typical lysosomal enzyme. During this process non-stoichiometric quantities of iron that amount to 5–10% of degraded hemoglobin, appear in the incubation medium, apparently released from heme. To the extent that this *in vitro* system simulates the conditions encountered by the host cell cytosol in the parasite's food vacuole, this process could provide a clue to the origin of iron required for parasite development and propagation. Several reports indicated that the parasite does not depend on an extracellular supply of iron [38, 39] but the chemical inertness of ferriprotoporphyrin IX under physiological conditions conceptually precluded this abundant product of host cell cytosol digestion as a possible source for iron. While QCDs have a marginal effect on the release of free iron upon acidification as such, they very efficiently inhibited the process in the presence of proteolytic activity (Fig. 3). Neither superoxide dismutase nor catalase had any effect either on hemoglobin decay or on the release of iron. This result indicates that oxidative radicals are not involved and alludes to the formation of ferryl radicals that are known to mediate the degradation of methemoglobin in an acid environment [40]. The mechanism of the inhibition of iron release by QCDs is presently under investigation. These observations, when considered in conjuction with the recently discovered irreversible effects on parasite growth exerted by a new class of iron chelators [41] and by deferroxamine [42], may explain the irreversible cytotoxic effects of QCDs mentioned above. However, in both cases, the puzzle of irreversibility remains to be resolved: it is hard to grasp why a temporary deprivation of iron supply, be it as detrimental to parasite growth as is possible, cannot be reversed after removal of the drugs with ensuing resurrection of the parasite.

Drug resistance

The global resurgence of malaria is due mainly to the advent of drug-resistant parasites and insecticide-resistant mosquito vectors. The evolution of drug resistance is inevitable under drug pressure but understanding its mechanistic details may provide a rationale for pharmacological intervention intended to prevent or reverse it.

In discussing drug resistance one should distinguish between two factors that may allow it to develop: (1) alterations in the as yet elusive drug target and

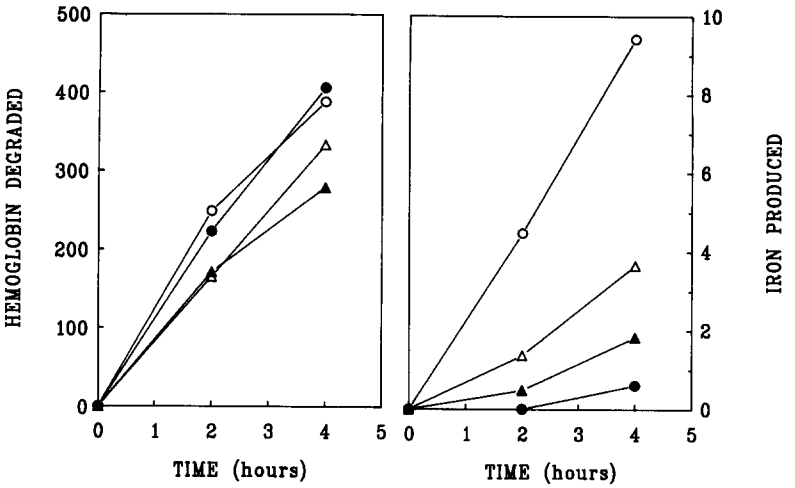

Fig. 3. Effect of QCDs on the degradation of hemoglobin and release of iron from heme. Normal red cell lysate was prepared by freezing and thawing a 50% suspension of cells and was diluted 1:10 into acetate buffer (20 mM Na-acetate, 150 mM KCl, pH 5) containing 0.5 mg/mL proteinase K and incubated at 37°. Samples taken at different time intervals were diluted 1:100 for the determination of absorbance at 414 nm and measurement of degradation of heme (left panel), and undiluted samples were assayed for free iron by the ferrozine method (right panel). Results are expressed in micromoles. QCDs were added at concentrations expected to be present in the parasite food vacuole when infected cells are exposed to drug levels causing *ca.* 50% inhibition of parasite growth. Control, (○); + 10 mM chloroquine, (●); + 1 mM quinine; (▲); + 0.3 mM mefloquine, (△).

(2) diminution in the capacity to accumulate the drug. There is ample evidence indicating that drug-resistant strains of the most lethal human malarial parasite *Plasmodium faciparum* accumulate less drug but this decrease in accumulation *per se* is not the sole factor in determining resistance [10]. It is premature to elaborate on possible changes in the drug target, simply because this has not yet been identified. However, we have shown previously that the vacuolar concentrations of chloroquine necessary to inhibit parasite growth increase with drug resistance, indicating a reduction in the susceptibility of the target to the drug [10].

Reduction of acidotropic drug accumulation is conceptually easier to fathom. Since the pH gradient is the driving force behind this effect, factors that regulate the vacuolar pH should be considered. As in any other known acidic organelle, the vacuolar pH is maintained by the balance between the proton pump activity that drives protons into the vacuole and the proton leak that provides a route for the exit of protons out of the vacuole along their concentration gradient. The former, whose presence has been demonstrated recently by the ATP-dependent acidification of isolated parasite vacuole [43, 44], should decrease and the latter should increase pH. Since biological membranes are usually tightly sealed to protons, an increased leak could be mediated by one of the known cation:proton antiporters (Na^+/H^+ or Ca^{2+}/H^+) but their presence in the vacuole membrane has not yet been detected [14]. However, repression of parasite growth by the Na^+/H^+ antiporter inhibitor amiloride and by diltiazem, which inhibits the Ca^{2+}/H^+ system, could suggest their important physiological role in the parasite, though not exclusively in the homeostasis of vacuolar pH (Krugliak, Nissani and Ginsburg, unpublished results).

In order to assess the involvement of the vacuolar proton pump in chloroquine accumulation, a kinetic model for drug accumulation able to discriminate between the contribution of H^+ pumping, H^+ leak and the putative "MDR pump" (see below), has been devised recently [45]. The model assumes that all three activities are located in the vacuolar membrane. While a previous report localized the MDR pump as being in the parasite cell membrane [46], recent evidence clearly restricts it to the vacuolar boundary [47]. The model suggests that kinetic analysis of chloroquine uptake can distinguish between factors that may affect drug accumulation. Thus, either increased H^+ leak or MDR pump should reduce steady-state drug accumulation and the half-life for drug influx. A weakened H^+ pump should not alter $T_{1/2}$ but will obviously reduce drug accumulation (Fig. 4). Analysing the kinetics of chloroquine uptake by about six drug-sensitive and -resistant strains of *P. falciparum* reveals that the resistant strains do indeed accumulate less drug, but they do so with the same half-time that their sensitive counterparts require to take up the drug [45]. Consequently, the vacuolar drug level aspect of chloroquine resistance must be related to a slower rate of H^+ pumping into the vacuole. At present, the basis of this condition is not known. In the quest for it, one could look into the down regulation of one of the subunits of the pump that would result in a smaller number of active pumps per vacuole; for a modulation of interaction(s) between subunits that could affect the pump's V_{max} or K_m values or into some cytosolic factor that might affect the pump's kinetic parameters. The latter mechanism,

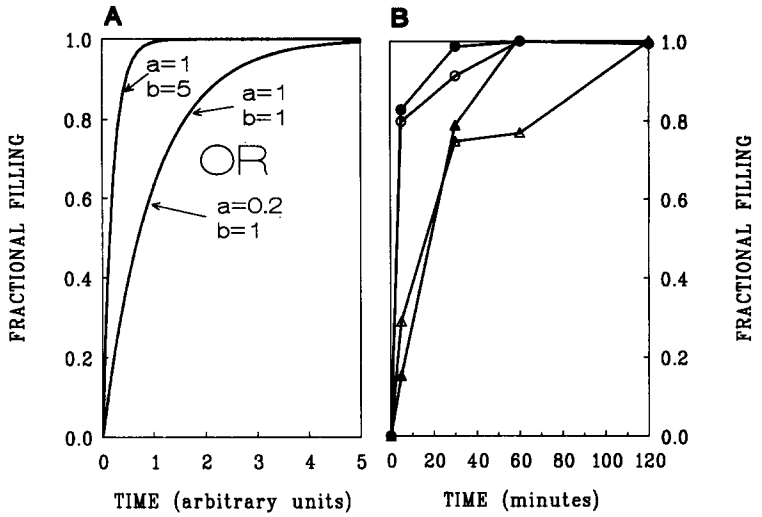

Fig. 4. (A) Theoretical time courses of uptake of chloroquine for drug-sensitive and various models of drug-resistant parasites. A weakened proton pump is expressed by reduction of a and b is increased if resistance is due to any mechanism that produces an increased efflux of accumulated chloroquine, such as proton leak or an MDR pump. The three cases depicted are where $a = b$, $a = 0.2$ and $b = 1$, and $a = 1$ and $b = 5$. The simulated data are plotted as amount of drug accumulated against time, normalized to its own steady-state level, lower of course in the drug-resistant cases. (B) Uptake of chloroquine as a function of time and concentration. Uptake of chloroquine into the chloroquine-sensitive strain FCN (filled symbols) and the resistant strain FCR_7 (empty symbols), as a function of time is plotted on the ordinate in terms of fractional filling. The external level of chloroquine was 10^{-7} (triangles) and 10^{-8} M (circles). Reproduced from *Biochemical Pharmacology* **41**: 1463–1470, 1991 [45] with permission.

if confirmed, identified and characterized, could definitely serve as a target for the reversal of drug resistance.

Reversal of drug resistance

Astute observers of scientific progress have noted apparent similarities between drug resistance in cancer cells and malarial parasites, in particular, the failure of the organism to accumulate the drug to the required toxic levels. They were, therefore, very encouraged when they found that compounds known to reverse drug resistance in cancer cells [48] were similarly effective in reversing chloroquine-resistance in drug-resistant strains of *P. falciparum*. Thus, in the presence of verapamil [49], methoxyverapamil, RO 11-2933/001 (tipamil analog), chloropromazine and its analog SKF 21133-A, diltiazem [50], imipramine and several of its analogs [51], and cycloheptadine [52], resistant *P. falciparum* strains become sensitive to chloroquine. All such enhancers are also potent inhibitors of parasite growth in culture. Concurrently, it was also shown that verapamil, diltiazem, vinblastine and daunomycin increase chloroquine accumulation, though never to the levels seen in the chloroquine-sensitive strains [53].

These observations lent support to a drug resistance mechanism that could be similar to that conjectured for MDR cancer cells, i.e. that reversing compounds inhibit an active drug efflux pump. To sustain this surmise, it was demonstrated that the half-time of drug release was considerably longer in chloroquine-sensitive parasites than in chloroquine-resistant strains [53] and it was, therefore, concluded that drug resistance results from an enhanced (active) drug extrusion that precludes the chloroquine-resistant parasite from accumulating the drug to toxic levels [53, 54]. While the effect of reversers on the antimalarial effect of chloroquine seems well founded, it is doubtful whether a direct mechanistic extrapolation can be made from MDR cancer cells to drug-resistant parasites. The kinetic model described above [45] predicts that the $T_{1/2}$ of chloroquine efflux should always be determined by the vacuolar drug level, regardless of the mechanism responsible for the poor accumulation. Consequently, the experimental foundation which underlies the supposition for an active drug efflux pump seems equivocal.

The following phenomenological differences that exist between drug-resistant parasites and cancer cells also allude to a different molecular mechanism. (a) Anticancer drugs enter cells slowly enough ($T_{1/2} > 1$ hr, [55]) to permit their efficient extrusion by means of an efflux pump. QCDs, on the other hand, enter into malaria-infected cells at least two orders of magnitude faster [10, 15, 56] and it is hard to conceive of a pump competent to deal with such rapid influx. (b) Metabolically deprived MDR cancer cells accumulate drug to the levels achieved in their drug-susceptible parent cell line [57, 58]. Under similar conditions malaria parasites are incapable of accumulation [59, 60], probably because no ATP is available to supply the vacuolar H^+ pump. (c) Unlike in MDR cancer cells, where they restore drug levels to those found in the drug-sensitive parent line [48], reversers in chloroquine-resistant malarial parasites confer full drug susceptibility but raise chloroquine

concentrations to only *ca.* 10% of those attained in susceptible strains [53]. (d) MDR cancer cells display cross resistance whereas chloroquine-resistant parasites in most cases are susceptible to the other QCDs quinine and mefloquine [61–64]. MDR characteristics in cancer cells have, thus, no analogy in malaria parasites. Significantly, penfluridol reverses mefloquine but not chloroquine-resistance, while the opposite is observed with chloropromazine, which affects a chloroquine-resistant strain but not a mefloquine-resistant strain [65]. These observations imply different drug resistance phenotypes for these two quinoline-containing analogs.

What does molecular biology tell us about drug resistance?

Drug resistance in cancer cells is associated with the amplification of MDR gene(s) and increased expression of their products [66]. No such connection could be observed in drug-resistant parasites, although *P. falciparum* contains two genes that are related to the mammalian MDR gene [67], one of which (*pfmdr*1) is markedly homologous to both the human and the murine MDR genes [68]. Its increased transcription in the chloroquine-resistant strain occurred only at the less sensitive schizont stage, while it did not take place at all at the most responsive trophozoite stage [20]. No relationship between the inheritance and amplification of either *pfmdr*1 or *pfmdr*2 genes and the chloroquine-resistant phenotype could be found [54]. Cloning and sequencing of the *pfmdr*1 gene revealed that resistant isolates differ by one to four nucleotides from chloroquine-sensitive genes in some strains, but were the same in others [69]. The unified message of the two latter reports [54, 69] is that *pfmdr* mutations are insufficient to confer the chloroquine-resistant phenotype.

Concluding remarks

There are indubitably two main take-home messages that emerge from this brief essay: we are still in the dark as to the ultimate target(s) of QCDs and, although we have some hints that drug resistance may result in part from decreased vacuolar H^+ pump activity, we have not yet started to grasp how this could occur. The reversal of drug resistance is definitely one of the most exciting advents of recent years, irrespective of its actual mode of action, i.e. whether it occurs by inhibition of an MDR pump or by synergizing the effects of QCDs. Understanding the mode of reversal at the molecular level may provide a basis for the rational design (or selection) of reversing compounds. Hopes for the rapid application of combination therapy are probably premature, since some such combinations increase demonstrably chloroquine toxicity to host cells [70].

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