REVERSAL OF DRUG-RESISTANT FALCIPARUM MALARIA BY CALCIUM ANTAGONISTS:
POTENTIAL FOR HOST CELL TOXICITY

by

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Abstract. Agents capable of reversing multidrug resistance (mdr) in falciparum malaria were investigated for potentiation of chloroquine accumulation and toxicity in a cell culture system. Verapamil, its analogue RO11-2933, and desipramine caused a dose-dependent increase in the accumulation of chloroquine within human and mouse hepatocytes but not within human lung cells. Only those cells in which drug buildup was enhanced by reversing agents tested positive for P-glycoprotein (PgP), the putative mediator of the enhanced drug efflux characteristic of mdr. Clinically achievable concentrations of verapamil (0.4 μM) and desipramine (1 μM) increased chloroquine accumulation within primary mouse hepatocytes by more than 50%. A well-differentiated "normal" human liver cell line (HepG2) was killed in media containing a combination of supraphysiological concentrations of chloroquine and verapamil but survived the same concentrations of either drug alone. Reversing agents may block FgP-mediated drug export from normal tissues as well as from mdr cells. Iatrogenic toxicity resulting from this buildup of potentially toxic drugs such as chloroquine within normal cells could complicate the in vivo reversal of mdr.

1. INTRODUCTION

The relentless spread of drug-resistant Plasmodium falciparum infections has stimulated interest in developing new approaches to malaria chemotherapy (Payne, 1987). The report by Martin et al. (1987) indicating that resistant falciparum parasites became sensitive to chloroquine in the presence of verapamil not only offered a new treatment strategy but also led to hope that the effectiveness of chloroquine - the most widely prescribed antimalarial (WHO, 1984) - might be retained.

Reversal of chloroquine resistance suggested underlying similarities between multidrug-resistant malaria and cancer. Multidrug resistance (mdr) in neoplastic cells is due to an enhanced drug efflux mechanism which prevents drug accumulation from reaching toxic levels in the cytosol of resistant cells (Slater et al., 1982; Rogan et al., 1984; Fojo et al., 1985). Certain calcium antagonists, phenothiazines, calmodulin inhibitors and tricyclic antidepressants - known collectively as reversing agents - reverse resistance by inhibiting this drug export process (Slater et al., 1982; Rogan et al., 1984; Fojo et al., 1985). Similarly, chloroquine-resistant malaria parasites are known to accumulate significantly less chloroquine than do susceptible parasites (Fitch, 1970), apparently by accelerating its release (Krogstad et al., 1987).

With the acquisition of mdr there is amplification of a family of genes - the mdr1 genes - which code for a membrane-associated glycoprotein (P-glycoprotein) (Chen et al., 1986). When cloned DNA for the human or mouse mdr1 gene is used to transfec previously sensitive cells, there is not only expression of P-glycoprotein (PgP) but these cells also display all the characteristics of the mdr phenotype, including reversal of resistance by calcium antagonists (Gros et al., 1986; Ueda et al., 1987). Recently, a P. falciparum mdr gene was also cloned and sequenced (Foote et al., 1989).

Unfortunately, PgP expression is not confined to drug-resistant cells. The use of monoclonal antibodies and measurement of mdr1 mRNA have demonstrated this protein in a variety of normal human tissues (Fojo et al., 1987; Thiebaut et al., 1987). If normal tissues use PgP to rid themselves of harmful substances, there might then be an accumulation of toxins inside the cytosol of normal cells when this excretory function is
blocked. It was therefore decided to study the effects of reversing agents on the accumulation of chloroquine and the anticancer drug, vincristine, within normal cultured cells.

2. MATERIALS AND METHODS

2.1 Drug accumulation

The accumulation of [3H]-chloroquine and [3H]-vincristine within primary mouse hepatocytes and three commercially available cell lines was measured in the presence of varying amounts of three reversing agents. In addition to verapamil, the present investigation also included R011-2933 (RO), a verapamil analogue (Kessel & Wilberding, 1985), and desipramine, a tricyclic antidepressant which reversed mdr in vitro and has been tested in monkeys infected with chloroquine-resistant P. falciparum (Bitonti et al., 1988).

Non-commercially available primary mouse hepatocytes were prepared as follows (Long et al., in press): freshly obtained livers from adult female Balb-C mice were perfused with a solution of 0.5% collagenase in oxygenated Hank's balanced salt solution (HBSS) to which ethylene glycol tetracetic acid (EGTA) and CaCl2 had been added. The perfused livers were teased in 2.5% fatty acid free bovine serum albumin solution in cold HBSS, filtered and centrifuged once at 50 x g for 5 minutes. Hepatocytes were then resuspended in culture medium and plated in 96-well microtitre plates at a density of 1 x 10^5 cells/cm².

Mouse hepatocytes were cultured in William's Medium E supplemented with 10% fetal bovine serum, 10 μg/ml insulin and 1 μM dexamethasone; the three commercial cell lines were cultured in minimal essential medium supplemented with 10% fetal bovine serum. Drugs dissolved in culture medium were added in triplicate to plated cells and incubated at 37°C in 5% CO2. At timed intervals, cells were washed three times with culture medium, detached from the bottom of the wells with 1X trypsin-EDTA, and radioactivity was measured by liquid scintillography. Results were expressed as the percentage increase in radioactivity in the presence of a reversing agent.

2.2 Staining for Pgp

Western blots of purified plasma membranes from cells used for accumulation studies (100 μg of protein/well) were probed with mouse monoclonal antibody (C219; Centocor-Malvern, PA, USA) against the cytoplasmic domain of Pgp using the technique of Kartner et al. (1985). Samples were solubilized with sodium dodecylsulfate (SDS)-urea sample buffer and processed on a 7.5% SDS polyacrylamide/4.5 M urea gel (100 μg of protein/well). Controls included blotted membranes plus second antibody alone and blotted membranes probed with an isotypically identical but irrelevant (anti-trypansomal) monoclonal antibody. Drug-sensitive KB3-1 and drug-resistant KB-V1 carcinoma cells were used as negative and positive controls, respectively.

2.3 Drug toxicity

Drug toxicity studies were done with a continuous culture of Hep-G2 cells (Whittaker M.A. Bioproducts Inc., Walkersville, MD, USA), a well-differentiated cell line sharing many characteristics with normal human hepatocytes (Knowles et al., 1980). Cells were plated in 24-well microtitre plates at 1 x 10^4 cells/well and incubated at 37°C in 5% CO2. After 4 days various concentrations of chloroquine and verapamil were added in duplicate - alone or in combination - and incubated for 60 h with 12 h changes of medium containing drug. The percentage of surviving cells was estimated blindly to the nearest 25% by cell morphology and by the ability of hepatocytes to extrude trypan blue.
3. RESULTS

3.1 Drug accumulation

The accumulation of [3H]-chloroquine and [3H]-vincristine within mouse hepatocytes rose with increasing concentrations of verapamil or R011-2933 after a 5 h incubation period (Fig. 1). Clinically achievable verapamil levels (0.4 μM) caused a 10% increase in vincristine and more than a 50% increase in chloroquine. Accumulation of chloroquine was enhanced by all reversing agents at all concentrations. Desipramine generated a 43%, 51% and 74% increase in chloroquine buildup within mouse hepatocytes at concentrations of 0.4, 1 and 10 μM respectively. These amounts of desipramine correspond to 104, 260 and 2600 ng/ml, and encompass the drug's therapeutic range in human patients (Amsterdam et al., 1980).

Both verapamil and R011-2933 also potentiated chloroquine and vincristine build up within Hep-G2 cells. Verapamil (5 μM) increased chloroquine by 52 ± 5% and vincristine by 280 ± 83%. The potentiation of intrahepatocyte drug accumulation induced by both verapamil and R011-2933 was sustained; increases were great after 24 h incubation as after 5 h. In marked contrast however, the addition of either verapamil or R011-2933 to the two human lung cell lines (MRC-5 and HEL-3; ATCC, Rockville, MD, USA) failed to increase chloroquine accumulation: there were no significant differences between counts obtained with reversing agents and those obtained without these agents.

3.2 Staining for Pgp

Pgp was demonstrated in the positive control, in mouse hepatocytes (Fig. 2), and in Hep-G2 cells (not shown). No bands were recognized in either of the lung cell lines or in the negative control (Fig. 2). Bands were absent in blots probed with second antibody alone and with an isotypically identical but irrelevant (anti-trypanosomal) monoclonal antibody.

3.3 Drug toxicity

The amount of chloroquine required to kill Hep-G2 cells was decreased by the addition of verapamil (Fig. 3). Cells remained viable following exposure to 44 μg/ml of chloroquine but 73% were killed when this amount of chloroquine was combined with 100 μM of verapamil. Neither 50 nor 100 μM of verapamil alone produced discernible toxicity.

4. DISCUSSION

Even though the toxicity of individual reversing and chemotherapeutic agents may be well described, there are no data on the potential deleterious effects of combining them. The list of drugs with known Pgp-blocking properties includes many pharmacological agents in widespread clinical use, such as verapamil and desipramine. Since Pgp is present in most human cells that serve a secretory or excretory function (Fojo et al., 1987; Thiebaut et al., 1987), the scope for dangerous drug interactions with host cells remains wide and undefined.

Each reversing agent tested produced a dose-dependent, sustained increase in the accumulation of chloroquine within Pgp-positive (Fig. 1) but not Pgp-negative cells. Desipramine and verapamil potentiated drug buildup within normal cells at concentrations achievable therapeutically. R011-2933 also potentiated accumulation, but the therapeutic range of this experimental drug has not yet been defined. No toxic effects were observed in hepatocytes during the accumulation experiments, but the long-term effects to a patient receiving verapamil for hypertension and chloroquine for malaria prophylaxis are unknown.

Deleterious effects were readily seen in Hep-G2 liver cells when supraphysiological concentrations of chloroquine and verapamil that were nontoxic individually were combined in vitro (Fig. 3). Chloroquine does not cause hepatotoxicity with normal clinical use (Zimmerman, 1978), and chloroquine levels over 50 times as high as peak therapeutic
levels were required to produce cell death. Therefore, the consequences of a 50% increase in drug accumulation may be even more relevant in tissues with an inherent susceptibility to being damaged by these compounds.

The physiological function of Pgp is unknown, though the results of the present study are consistent, with reversing agents blocking Pgp-mediated excretion of toxins from normal cells. Consequently, accumulation of potentially dangerous drugs could be even more prominent in cells rich in this protein. Human adrenal medulla, for example, contains more than 20 times as much Pgp as does human liver (Pejo et al., 1987). More needs to be learned about the physiological role of Pgp and the consequences of disrupting it.

The results of the present study indicate that combination treatment for mdr may not target drug-resistant parasites as specifically as had been expected. As suggested by Gottesman & Fastan (1988) and shown by our data, this therapeutic modality is a double-edged sword. Chloroquine has considerable toxic potential and was concentrated within Pgp-containing normal cells by reversing agents. Iatrogenic toxicity could therefore complicate the in vivo reversal of mdr and should be taken into consideration by physicians contemplating clinical trials.

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RESUME

INVERSION DE LA RESISTANCE AUX MEDICAMENTS DU PALUDISME
A FALCIPARUM PAR DES ANTAGONISTES DU CALCIUM :
ACTION POSSIBLE SUR LA TOXICITE CELULLAIRE DE L'INFECTION

Des agents capables d'inverser la résistance à la pharamcothérapie du paludisme à falciparum ont été examinés pour potentialisation de l'accumulation de chloroquine et de la toxicité dans un système de culture cellulaire. Le vérépamil, son analogue RO11-2933, et la désipramine ont provoqué une augmentation dose-dépendante de l'accumulation de chloroquine dans des hépatocytes humains et de la souris, mais non dans des cellules pulmonaires humaines. Seules les cellules dans lesquelles l'accumulation du médicament était augmentée par les agents d'inversion ont été trouvées comme étant positives pour la P-glycoprotéine (Pgp), le médiateur supposé de la perte augmentée de médicaments dans la résistance à la pharmacothérapie. L'obtention en clinique de concentrations de vérapamil (0,4 μM) et de désipramine (1 μM) augmentait l'accumulation de chloroquine dans les hépatocytes de la souris de plus de 50 %. Une lignée de cellules hépatiques humaines "normales", bien différenciées (Hep-G2), étaient tuées dans un milieu contenant une association de concentrations supraphysiologiques de chloroquine et de vérapamil, mais survivaient aux mêmes concentrations de chacun des médicaments donnés isolément. Les agents d'inversion peuvent bloquer la perte de médicaments à médiation par la Pgp, que ce soit depuis des tissus normaux ou de cellules résistantes à la pharmacothérapie. Une toxicité iatrogène résultant de cette accumulation de médicaments potentiellement toxiques, comme la chloroquine, dans des cellules normales pourrait compliquer l'inversion in vivo de la résistance à la pharmacothérapie.
REFERENCES


FIG. 1

Primary mouse hepatocyte accumulation of [3H]-chloroquine (solid lines) and [3H]-vincristine (dotted lines) added alone or in combination with a calcium antagonist to culture medium for 5 hours. Values obtained from four experiments are expressed in mean percentage increase ± standard error of the mean.
FIG. 2

Western blots of purified plasma membrane vesicles from A) MRC-5 lung cells, B) HEL-3 lung cells, C) Primary mouse hepatocytes, D) Negative control: drug-sensitive KB-3-1 carcinoma cells, E) Positive control: multidrug-resistant KB-V1 cells. Blots were probed with a monoclonal antibody (C219) directed against the cytoplasmic domain of P-glycoprotein.
FIG. 3

Percentage of Hep-G2 human liver cells killed (to the nearest 25%) after 60 hours of incubation with chloroquine alone (µg/ml of free base) or in combination with verapamil.