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TRANSIENT ELECTROPERMEABILIZATION OF CELLS IN CULTURE

INCREASE OF THE CYTOTOXICITY OF ANTICANCER DRUGS

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Abstract—The electropermeabilization (EPN) of living cells allows the uptake of non-permeant molecules and can reveal their potential activity on cells without the constraints of the plasma membrane crossing. We decided to compare the cytotoxicity of some anticancer drugs on electropermeabilized (EP) and non-permeabilized (NEP) cultured DC-3F cells exposed to the drugs for a short time. After EPN, the increase in cytotoxicity varies between 1 and more than 700 times, depending on the usual cell uptake pathway of a given drug. The most relevant increase of toxicity was observed with molecules such as netropsin (200-fold) and bleomycin (700-fold) which in ordinary conditions weakly diffuse through the plasma membrane. Only a 3–5-fold increase of the cytotoxicity was observed with lipophilic drugs able to rapidly diffuse through the plasma membrane (actinomycin D, NMHE) both in the case of drug-sensitive and resistant cell strains. This increased toxicity is clearly related to a facilitated uptake because, after electropermeabilization, the effects of melphalan (a drug which enters intact cells via leucine transporters) are not modulated by the external leucine concentration. Thus, EPN enables us to reveal the intrinsic toxicity of hydrophilic molecules which have a limited access to their intracellular targets. We propose that EPN can be used as a novel screening procedure of new cytotoxic molecules which could be modified thereafter in order to facilitate their cellular uptake.

When a cell is exposed to short and strong electric pulses, its membrane undergoes a remodelling process characterized by the occurrence of transient permeation structures ("electropores") [1, 2]. If the strength of the external electric field is properly chosen, it is possible to permeabilize most of the shocked cells while preserving the viability of the cell population [3]. Electropermeabilization has been used for investigations concerning the crossing of the plasma membrane: introduction of exogenous molecules into the cells [3–11], study of the exocytotic mechanisms and the role of the G-proteins in the secretion [12, 13], distinction between the pinocytosis-mediated and the diffusion-mediated uptakes of molecules such as sucrose or raffinose [14, 15]. So far, most of the reported work has been concerned with the introduction of genetic material (DNA or RNA) into living cells [4–8]. However, at least three laboratories have already used this technique in order to enhance to some extent the uptake of drugs [16–18]. We decided to explore the possibilities offered by the EPN§ of living cells with a generator which delivers reproducible, easily monitored square-wave electric shocks adapted to each cell type. Here we report our observations on a large spectrum of anticancer and cytotoxic drugs assayed with and without EPN on drug-sensitive as well as drug-resistant cell strains and we show that this technique enables us to reveal the intrinsic cytotoxicity of little permeant hydrophilic molecules.

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§ Abbreviations used: EPN, electropermeabilization; EP cells, electropermeabilized cells; NEP cells, non permeabilized cells; NMHE, 2-*N*-methyl-9-hydroxy-ellipticinium; MTX, methotrexate; MLP melphalan; MTM, mithramycin; NTP, netropsin; BLM, bleomycin; LY, lucifer yellow; PAP, *Phytolacca americana* (Pokeweed) antiviral protein; EC_{50}, dose which reduces the cloning efficiency to 50% of the control.

MATERIALS AND METHODS

Chemicals and electrical device. 2-*N*-methyl-9-hydroxy-ellipticium and 9-hydroxy-ellipticine have been kindly provided by Dr. E. Lescot (Villejuif, France), the 30,000 daltons *Phytolacca americana* (Pokeweed) antiviral protein by Dr. S. Junqua (Villejuif, France) (Junqua, S. *et al.*, in preparation) and didemnin B (solubilized in DMSO, 1% final) by Pr. B. Castro (Montpellier, France). Actinomycin D (Lyovac Cosmegen, dactinomycin, M.S.D. Lab., U.S.A.), melphalan (Alkeran, Wellcome Lab., U.K.), methotrexate (Méthotrexate, Lab. Roger Bellon, France), bleomycin, (Bléomycine, Lab. Roger Bellon, France), netropsin (Serva) and Lucifer Yellow CH (Sigma) are dissolved in PBS.

We used the "electropulsator" commercially available from BIOBLOCK (Strasbourg, France). The output voltage from this generator is comprised between 0 and 1000 V and is maintained for periods of time ranging from 5 to 150 μsec. This square wave signal can be delivered either *ad libitum* or at a

preprogrammed frequency (ranging from 0.1 to 10 Hz). The efficiency of the impulsions is checked through an oscilloscope connected to the generator.

Cells. DC-3F cells, a spontaneously transformed Chinese hamster lung fibroblast [19], and the derived cell sublines DC-3F/AD X [19] as well as DC-3F/9-OH-E 0.6 clone F [20], resistant respectively to actinomycin D and to 9-hydroxy-ellipticine, were cultured in MEM supplemented with 8% fetal calf serum, 2 mM L-glutamine, penicillin and streptomycin. The DC-3F/AD X cells display a higher unidirectional drug efflux due to the amplification and overexpression of the P-glycoprotein coding genes [21]. The DC-3F/9-OH-E 0.6 cells have a modified "target", the topoisomerase II, but under our operating conditions, and in more classical assays [22], these cells have also revealed cross-resistance against actinomycin D. These cells could also have a membrane resistance mechanism similar to the gp 170-dependent active efflux of other actinomycin D-resistant cell lines. The NIH 3T3-A 11 clone derived from the NIH 3T3-A cells (Dr. J. F. Nicolas, Institut Pasteur, Paris) was used for the testing of PAP toxicity [3].

To measure the relative cloning efficiency of the DC-3F and related cells, dilutions of the cells were seeded in triplicate in complete culture medium (500 cells per 60 mm diameter cell culture dish for the DC-3F cells and 700 cells for the other strains). Colonies were counted after 5 days (DC-3F cells) or 7 days (DC-3F/AD X and DC-3F/9-OH-E 0.6 cells) of culture. The values obtained were expressed as the percentage of the number of clones obtained with control cells treated in absence of drug. The absolute cloning efficiency of the controls was of the order of 80%. Each drug was tested at least twice.

Electropermeabilization conditions. After trypsination of exponential growing cells and inactivation of trypsin by complete medium, cells were washed twice in 0.5 mM Ca^{2+} supplemented *S*-MEM (without serum), and resuspended in the same ice-cooled medium at a density of 1.1×10^7 cells/ml. Aliquots of 0.09 ml of cell suspension were mixed with 0.01 ml of 10-fold concentrated drug solution, and immediately exposed to the electric treatment (8 impulsions of 0.1 msec at a frequency of 1 Hz and at the desired intensity). After appropriate post-shock incubation, cells were diluted about 10^4 times and plated in culture dishes for the determination of the cloning efficiency. NEP cells were treated similarly except for the electric field exposure.

RESULTS

Viability and permeabilization of the shocked cells

We determined the optimal electrical shock conditions using the "Lucifer Yellow (LY) test" that we have recently described [3]. Cells were shocked at different field strengths, incubated at 4° for 5 min and at 37° for 20 min in the presence of 1 mM external LY. After two washings, we scored the percentages of: (1) living, nonfluorescent thus non-electropermeabilized (NEP) cells; (2) living, homogeneously and highly fluorescent thus electropermeabilized (EP) cells; (3) dead, low and granular fluorescent cells. The DC-3F cells and derived

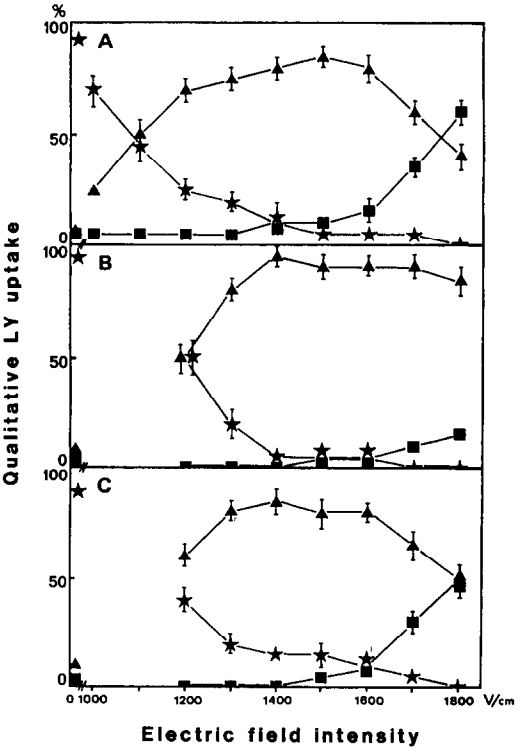

Fig. 1. Lucifer yellow assay: determination of the electric field intensity allowing the highest percentage of permeabilized, living cells (triangles) to be obtained. The percentage of non-permeabilized (stars) and of dead cells (squares) have also been reported. (A) DC-3F cells; (B) DC-3F/AD X cells; (C) DC-3F/9-OH-E 0.6 cells.

cell lines DC-3F/AD X and DC-3F/9-OH-E 0.6 behave in a similar fashion (Fig. 1). We chose to treat all the cells at 1500 V/cm, for which a maximum (between 80 and 90%) of cells are permeabilized and survive while a minimum (less than 10%) of cells are killed during the treatment. At this field intensity, the lethal effect observed seems to be due to the manipulation of the cells (detachment using trypsine, washings) more than to the electric shock itself.

Determination of the shock operating conditions

For substances such as LY, the pokeweed antiviral protein (PAP) or DNA, unable to cross the plasma membrane (non-permeant molecules), we currently maintained the cells at 4° for 5–10 min after the shock and then we transferred them at 37° for 20 min before diluting or handling the cells. With compounds able to flow through the plasma membrane (either by passive diffusion through the lipid bilayer or channels, either by active systems such as pumps or transporters), our main concern was to reduce the pre- and post-shock incubations times in the presence of the drugs in order to improve the differences between the "pore-mediated" uptake and the normal "trans-membrane" uptake. First, adding the LY after the electric treatment, we checked the kinetics of the "electropore" closure at different temperatures (data not shown). Second, in preliminary experiments using actinomycin D, we compared some time/temperature couples of conditions in order to improve

the different toxicity of the drug upon electropermeabilized and non-permeabilized cells. Cloning efficiency of the shocked cells was not dependent on the temperature of the post-shock incubations. This led us to perform the experiments reported here under the following conditions: the cells (10^7/ml) are chilled at 0–4°, mixed to the drug, submitted to 8 impulsions of 100 μsec and 1500 V/cm at a frequency of 1 Hz, incubated for 5 min at 25°, diluted at least 10,000 times, counted and distributed in Petri dishes for the cloning efficiency assay.

Effects of the actinomycin D treatment

In the case of the sensitive DC-3F cells, the electropermeabilization allows a 2.7-fold factor of reduction of the EC_{50} from $(27.5 \pm 10) \times 10^{-6}$ M to $(10 \pm 2) \times 10^{-6}$ M (Fig. 2). In the case of the resistant DC-3F/AD X cells and in the same conditions, it was impossible to detect any cytotoxic effect (Fig. 2). The DC-3F/9-OH-E 0.6 cells displayed a relative resistance to actinomycin D as compared to the DC-3F cells. In this case, the electropermeabilization increased the toxicity by a factor next to 2.3, decreasing the EC_{50} from $(80 \pm 10) \times 10^{-6}$ M to $(35 \pm 5) \times 10^{-6}$ M (Fig. 2). Nevertheless, it was possible to partially reverse the resistance of the EP DC-3F/AD X cells by supplementing with 10^{-5} M verapamil both the medium of shock and the medium of culture of the plated cells ($EC_{50} = (4 \pm 1) \times 10^{-4}$ M) (data not shown). This could not be achieved if verapamil was present only during the shock and the post-shock incubation, and then diluted at the same time as the cells and the actinomycin D.

Effects of the NMHE treatment

In the case of the sensitive DC-3F cells, the EC_{50} of the EP cells ($(1.5 \pm 0.3) \times 10^{-4}$ M) was four times lower than the EC_{50} of the NEP cells ($(6 \pm 0.6) \times 10^{-4}$ M) (Fig. 3). In the case of the DC-3F/AD X cells and of the DC-3F/9-OH-E 0.6 cells, for both the EP and the NEP cells, no significant toxicity was detected in the same experimental conditions, at least up to 10^{-3} M.

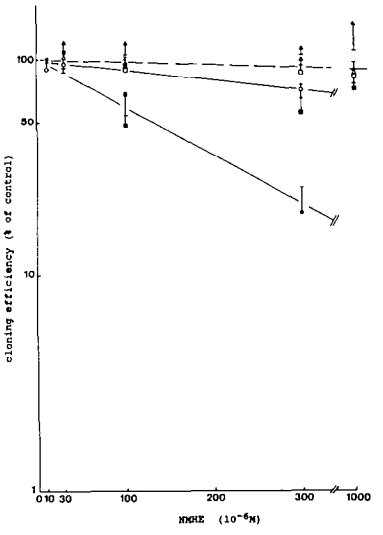

Fig. 3. Effects of the NMHE treatment. Electropermeabilized (closed symbols) and non-permeabilized (open symbols) DC-3F (circles), DC-3F/AD X (triangles) and DC-3F/9-OH-E 0.6 (squares) cells were treated with NMHE under our standard conditions.

Effects of the melphalan treatment

We have compared the toxicity of the melphalan (MLP) upon the DC-3F cells in three different media (Fig. 4). In the normal 0.5 mM Ca^{2+} supplemented

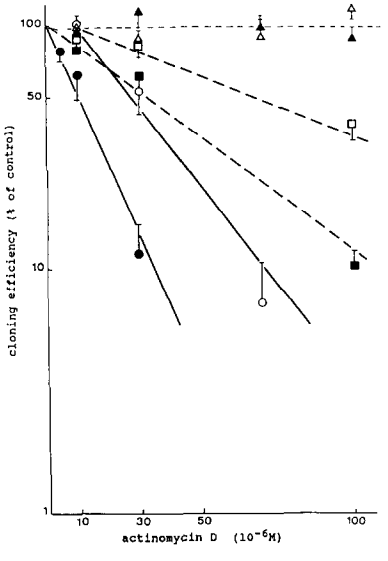

Fig. 2. Effects of the actinomycin D treatment. Electropermeabilized (closed symbols) and non-permeabilized (open symbols) DC-3F (circles), DC-3F/AD X (triangles) and DC-3F/9-OH-E 0.6 (squares) cells were treated with actinomycin D under our standard conditions.

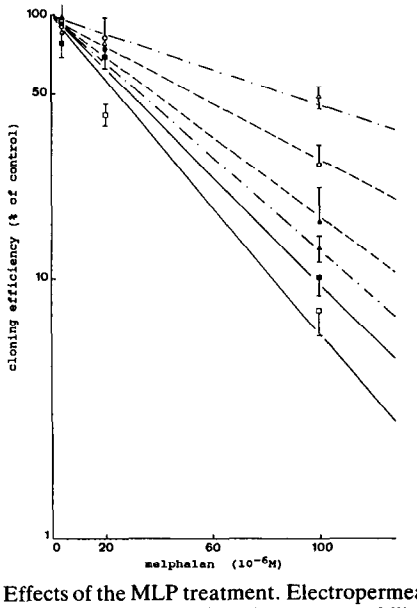

Fig. 4. Effects of the MLP treatment. Electropermeabilized DC-3F cells (closed symbols) and non-permeabilized cells (open symbols) were treated either in the normal shock medium (circles) or in the same medium but supplemented with 1.5 mM leucine (triangles) or further in the amino acid-free PBS (squares).

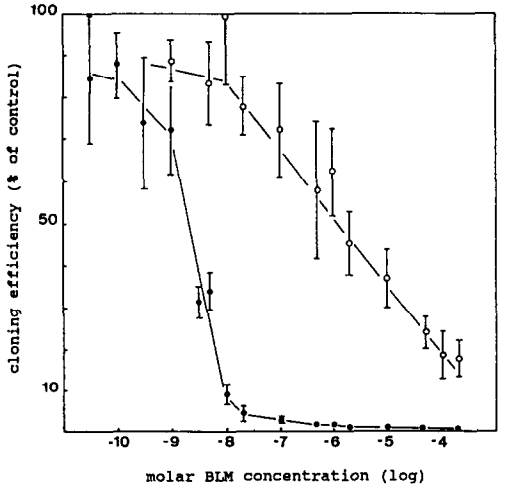

Fig. 5. Effects of the BLM treatment. Electropermeabilized (closed symbols) and non-permeabilized (open symbols) DC-3F cells were treated with the BLM under our standard conditions.

S-MEM medium, containing 0.3 mM of leucine, the EC_{50} of the NEP cells was $(5.5 \pm 1) \times 10^{-5}$ M. In the same medium further supplemented with leucine to achieve a 1.8 mM concentration, their EC_{50} was $(9.5 \pm 1) \times 10^{-5}$ M, i.e. nearly twice as high. In isoosmotic leucine-free PBS (phosphate buffered saline), their EC_{50} was $(2.5 \pm 0.5) \times 10^{-5}$ M, i.e. half of the EC_{50} measured in our usual shock medium. In contrast, when the cells are electropermeabilized, there were no significant differences in function of the leucine content of the medium. Their EC_{50} (near 3.5×10^{-5} M) was almost as low as in the case of the unshocked cells in PBS.

Effects of the netropsin and the bleomycin treatments

In the case of the netropsin (NTP), the difference between the EC_{50} of the EP DC-3F cells

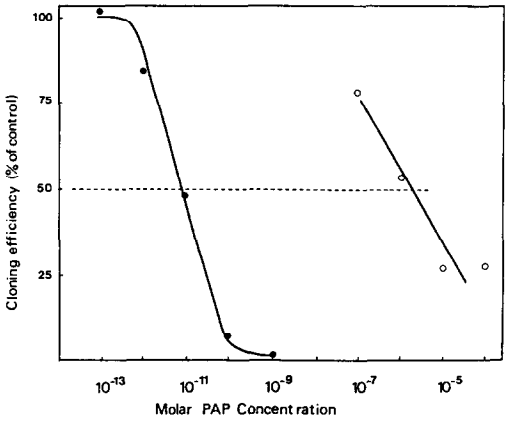

Fig. 6. Effects of the PAP treatment. Electropermeabilized (closed symbols) and non-permeabilized (open symbols) NIH 3T3 cells were treated with the PAP using the same procedure as for the DC-3F cells except that the electric field intensity applied was 1400 V/cm (the optimum electric field intensity for this cell type has also been determined using the lucifer yellow test).

$((1.6 \pm 0.2) \times 10^{-5}$ M) and the EC_{50} of the NEP cells $((3.2 \pm 0.5) \times 10^{-3}$ M) shows that EPN can reveal an increase of about 200 times the toxicity of this compound. In the presence of bleomycin (BLM), the EC_{50} of the EP DC-3F cells $((1.5 \pm 0.5) \times 10^{-9}$ M) was very low in respect to the EC_{50} of the NEP cells $((1 \pm 0.4) \times 10^{-6}$ M) (Fig. 5). For comparison with a non-permeant, highly cytotoxic substance (see Discussion), we also report in Fig. 6 an unpublished representation of a previous result of our laboratory: the toxicity of the PAP upon EP and NEP cells. For both the BLM and the PAP, the range of the tested concentrations was so extended that the use of logarithmic abscissa was necessary.

Effects of other drugs

In the presence of methotrexate, up to 2×10^{-4} M, we did not detect any cytotoxicity of the sensitive DC-3F cells. The same result was obtained with the 9-OH-ellipticine (tested only at concentrations up to 25×10^{-6} M), and with the didemnin B, (up to 3×10^{-6} M). When the cells were in permanent presence of the product, didemnin B almost completely inhibited the growth of the DC-3F cells at 10^{-8} M.

DISCUSSION

The antitumoral drugs enter the intact cells more or less easily, and all are able to cross the plasma membrane. Nevertheless, the uptake mechanisms display important differences. Actinomycin D, the ellipticines, and, in general, lipophilic molecules enter cells by passive and rapid diffusion. Drugs such as melphalan or methotrexate cross the membrane through active transporters (an amino acids transporter in the case of the MLP [23] and a folic acid transporter in the case of the MTX [24]). The mechanism of bleomycin uptake is not clear, however, only a very little amount of the external drug enters the intact cells [25]. It is generally admitted that BLM crosses the membrane by slow passive diffusion [26]. Molecules such as some vegetal toxins [27, 28], unable to cross the plasma membrane (non-permeant molecules) can cause very restricted lethal effects even if their intrinsic cytotoxic capacities are very high as revealed after electropermeabilization [3].

One of the purposes of this work was the determination of relationships between the drug uptake mechanisms and the enhancement of the cytotoxicity due to the electric field-induced reversible permeabilization of living cells.

In order to differentiate the facilitated uptake of the electropermeabilized cells from the normal uptake of the non-permeabilized cells, we reduced as much as possible the incubation times of the cells in the presence of drugs. Therefore, we examined the kinetics of the closure of the reversible structures of permeation induced by the electric field (data not shown) and limited the time of the cells/drug contact to the time necessary to the membrane resealing. For this reason, we applied to the cells relatively short treatments (the cells remained in the presence of the drug for no more than 6 min) as compared to classical treatment times (from 1 hr until 72 hr).

Consequently, our short treatment conditions were inadequate for testing molecules possessing a very low intrinsic cytotoxicity. For example, in the case of the methotrexate (MTX), even if all the molecules of dihydrofolate reductase (DHFR) are inactivated during the treatment, this one is too short for inducing an effect on the synthesis of new DHFR molecules and on their ulterior inactivation.

After electropermeabilization, drugs uptake is actually and predominantly diffusion free, i.e. almost independent of the normal "membrane-mediated" ways of plasma membrane crossing. On intact cells, the toxicity of the MLP depends on amino acids concentrations in the incubation medium. This is related to the competition between some amino acids and the MLP for the same membrane transporters [23]. On the contrary, after EPN, MLP is almost as cytotoxic whatever the amino acids concentration may be. We conclude that: (1) the uptake becomes (at least partially, but rather principally) independent of the mechanisms of the plasma membrane crossing, and, according to our knowledge, "pore-mediated" [1–3, 29, 30]; (2) in the presence of high external amino acid concentrations, the phenotypic resistance to the MLP results from the reduction of the unidirectional influx of the drug, and we assume that EPN re-establishes MLP cytotoxicity by transitorily restoring the unidirectional influx.

We also compared the cytotoxic responses of drug sensitive (DC-3F) and resistant (DC-3F/AD X and DC-3F/9-OH-E 0.6) cell lines to both actinomycin D and NMHE. With respect to the DC-3F sensitive cells, DC-3F/AD X cells display a higher unidirectional drug efflux due to the amplification and overexpression of the P-glycoprotein coding genes [21]. The electropermeabilization is unable to reverse the drug resistance of these cells. This result was expected because: (1) the electric shocks only transitorily modify the permeation capacities of the plasma membrane; (2) the drug efflux is supposed to exclude the drug whatever the influx mechanism may be (membrane-mediated or pore-mediated diffusion); (3) even in the absence of electric treatment, the actinomycin D and NMHE unidirectional influxes are important. Thus, the net influx cannot be very enlarged in spite of an increase of the unidirectional influx.

Actinomycin D efflux from the DC-3F/AD X cells can be reduced if cells are treated and then cultured in the presence of 10^{-5} M verapamil. In these incubation conditions (but, nevertheless, at higher external actinomycin D concentrations than in the case of the sensitive DC-3F cells), EP cells but not NEP cells can uptake (and furthermore retain for a sufficiently long time) an amount of drug sufficient to cause a clear reduction of their survival. Thus, the differences observed between the EP and NEP sensitive DC-3F cells can be qualitatively reproduced in the case of the DC-3F/AD X cells.

We cannot exclude the possibility that the stress induced by the electric pulses could modify the mechanisms of drug cytotoxicity. Nevertheless, our observations are in complete agreement with the fact that EPN can facilitate the uptake of cytotoxic compounds. For each of the drugs tested, two characteristics have to be compared: on one hand, the potentiation of their cytotoxicity which is obtained after the electropermeabilization of the cells; on the other hand, the barrier effect of the cell membrane against the uptake of the studied compound, as it has been reported until now.

If the drug crosses the plasma membrane mainly transported by active carriers (MLP, MTX), the membrane does not constitute a barrier for its uptake. This is demonstrated by the fact that EPN cannot increase its cytotoxicity, except in conditions which slow down the drug-carriers activity (as for example, the excess leucine concentration in the medium, in the case of the MLP) and, thus, the unidirectional influx of the drug.

In the case of the lipophilic substances which rapidly diffuse through the plasma membrane (9-OH-ellipticine, NMHE, actinomycin D), the membrane crossing is a step only weakly limiting the access to their intracellular target. Both unidirectional influx and efflux are high. In fact, these substances do not display a very high intrinsic toxicity and their activity is related to the fact that they can accumulate in high amounts in intact cells. During our short treatments, we were obliged to use high concentrations of these products and only weak differences were observed between the NEP and the EP cells (toxicity increase: $\times 2.3$ to $\times 4$). This is similar to the result published by Melvik *et al.* [17] and concerning another member of this class of antitumoral agents, the cis-platinum.

For the hydrophilic substances diffusing slowly through the plasma membrane (BLM and NTP), the membrane is a strongly limiting step for the access to their intracellular target. Unidirectional influx is very weak. NTP is almost inactive on intact cells, but a toxicity increase of 200 times is observed after electropermeabilization. NTP becomes as toxic as MLP, actinomycin D or NMHE due to a rapid accumulation of the drug allowed by the rise in the unidirectional influx. On the contrary, BLM is already efficient on intact cells at low concentrations because it has a very high intrinsic toxicity. BLM produces DNA breaks and, moreover, displays a catalytic mode of action [26] and thus does not need to accumulate to much extent into the cells. BLM killed EP cells at the nanomolar range. This presents a reduction of 700 times of the EC_{50} of the non-permeabilized cells. The difference is even greater at the EC_{37} or the EC_{10} levels of toxicity (manuscript in preparation).

An extreme situation among substances belonging to the second class of the hydrophilic molecules is revealed by the *Phytolacca americana* antiviral protein (PAP). This natural antiviral protein non-toxic for its host and producer, the pokeweed, does not cross the plasma membrane at all. It enters the cells only by pinocytosis and it is an example of what we call non-permeant substances [3]. The intrinsic PAP toxicity is extremely high: one or a few molecules in the cell cytosol are sufficient to kill the cell, because of a catalytic inactivation of the 60 S ribosomal sub-units [27, 28]. In fact, PAP, like BLM, irreversibly damages a nucleic acid: the ricin A-chain-like toxins cleave a *N*-glycosidic bond in a particular position of the 28S rRNA [31, 32]. Due to the absence of a transmembrane influx, PAP is moderately toxic

for the intact cells but its toxicity is increased 2.10^5 times after EPN of the cells. It has to be noticed that the cytotoxicity plots concerning the BLM and the PAP present striking similarities which will be discussed elsewhere (manuscript in preparation).

Therefore, in the light of the differences observed with these drugs and from the previously discussed results concerning the cases of genotypic or phenotypic drug resistance, it appears that the more the cell membrane acts as a barrier for the unidirectional influx of a compound, the more the electropermeabilization allows a gain of toxicity.

On the other hand, the more the cytotoxicity of a new compound is increased after EPN, the more this product possesses a reduced unidirectional influx through the cell membrane in physiological conditions. In front of a new product which strongly inhibits the growth of the cells, EPN allows discrimination between either an activity due to a great accumulation or due to a great intrinsic toxicity. Such was in fact the case for the didemnin B [33] (ID_{50} = 1 nM for the L1210 cells) [34]. As with this compound we did not detect major differences in the toxicity after the electric shocks, we can propose that the toxicity of the didemnin B is due to a rapid influx allowing the internalization of large amounts of this substance. This is quite in line with the lipophilic nature of this molecule.

Thus, the electropermeabilization seems to be an interesting system highly useful for testing the intrinsic cell toxicity of new molecules, such as, for example, the sequence-specific DNA binding molecules designed by Dervan [35]. Furthermore, EPN could be used as a screening procedure useful to discover non-permeant intrinsically highly cytotoxic molecules which could be then rendered permeant by chemical modifications. Ideal molecules would be able to produce irreversible damages on cell critical targets (such as the nucleic acids) by catalytic mechanisms.

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