Uptake, Cytofluorescence, and Cytotoxicity of Oxazolopyridocarbazoles (Amino Acid-Ellipticine Conjugates) in Murine Sarcoma Cells

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ABSTRACT

The uptake, cytofluorescence, and cytotoxicity of elliptinium (NMHE) and a series of fluorescent oxazolopyridocarbazoles [amino acid-ellipticine conjugates (AA-NMHE)] were studied in murine sarcoma cells. For all these drugs, the uptake was rapid, directly proportional to the drug concentration, and unaffected by metabolic inhibitors which is consistent with a diffusion mechanism. By 4 h, the intracellular concentration of NMHE exceeded the external drug concentration by about 100 times; this suggests that the toxicity of NMHE is not, as previously assumed, limited by its transport across tumor cell membranes. Conjugation of NMHE with aliphatic amino acids increased the cellular uptake 5- to 7-fold. Cellular exposure to AA-NMHE conjugates resulted in the appearance of granular cytoplasmic fluorescence which was readily translocated to the nucleus upon continued exposure to fluorescent light. The cytotoxicity of the AA-NMHE conjugates (drug concentration required to reduce colony formation by 63% on the exponential part of the survival curve = 3–14 μM) was less than that of NMHE (drug concentration required to reduce colony formation by 63% on the exponential part of the survival curve = 0.7 μM) as shown by colony formation following 4 h drug exposure. In contrast, the isoleucine-NMHE conjugate was the most cytotoxic compound (drug concentration required to reduce colony formation by 63% on the exponential part of the survival curve = 0.045 μM) when the drug exposure period was extended to 8 days. The general lower toxicity of the AA-NMHE conjugates is likely due to loss of the phenolic character of the NMHE moiety; therefore, attempts to link NMHE to amino acids remain attractive but will have to be done without affecting the 9-hydroxy group of NMHE.

INTRODUCTION

Ellipticine and several of its analogues have shown anticancer properties in various animal tumor models (1) and in man (2, 3). Among these analogues, NMHE has been used in the treatment of renal carcinomas, soft tissue sarcomas, and especially, advanced breast cancer (4).

It is possible that one of the limiting factors in the anticancer activity of ellipticine drugs is the transport of these drugs across tumor cell membranes. In this respect, it should be noted, that NMHE is a large, charged, and hydrophilic drug consequently exhibiting the most unfavorable structures in terms of membrane transport (5). In order to increase drug uptake a series of fluorescent oxazolopyridocarbazoles (AA-NMHE; Fig. 1) were prepared by taking advantage of the ability of the oxidized form of NMHE to undergo nucleophilic addition with nitrogen do

Since the uptake of AA-NMHE by tumor cells has not previously been investigated, we studied a series of AA-NMHEs in order to define the relationship between structure and cellular uptake. By taking advantage of the fluorescent properties of AA-NMHE, we have also followed the appearance of cellular fluorescence and attempted to establish a relationship between cellular uptake of AA-NMHE, their cellular distribution, and their cytotoxic properties.

MATERIALS AND METHODS

Cells and Culture Medium. The murine sarcoma cell line, TBL CL2 (8) was maintained as a monolayer culture in Eagle’s MEM supplemented with 8% FCS, streptomycin (100 μg/ml), and penicillin (100 IU/ml). Under these conditions, the cells have a population-doubling time of 18–19 h.

Chemicals. NMHE was provided by Sanofi Co. (Sisteron, France). The oxazolopyridocarbazoles were synthesized from NMHE and aliphatic amino acids (Gly, Ala, α-amino butyric acid, Val, Leu, and lle) as described (6, 7). All the AA-NMHEs bind tightly to naked DNA in vitro with about the same affinity constants as previously reported for NMHE (3, 9). Radiolabeled AA-NMHEs were synthesized in a similar manner from 14C-labeled amino acids (CEA, Gil-sur-Yvette, France), which resulted in conjugates with a specific activity of approximately 1.0 μCi/mmol. Drug purity was at least 95% as shown by high performance liquid chromatography using a reverse phase column (C18-MBondapak; Waters) and an elution mixture composed of 80% methanol and 20% water containing sodium heptane sulfonate (1 g/liter) and acetic acid (2 ml/liter). The same technique was used to demonstrate that the drugs are stable in culture medium at 37°C for at least 24 h.

Drug Uptake. For kinetics experiments, the cells were plated on 25-cm² tissue culture bottles (NucIon, Roskilde, Denmark) containing 5 ml of growth medium about 18 h before the beginning of the experiments. The number of cells were adjusted to about 1 × 10⁶ cells/bottle at the time of drug exposure. At time zero the medium was replaced with 5 ml of MEM with 8% FCS containing 14C-labeled AA-NMHE (5 nmol/ml corresponding to about 2 ng/ml) and the cells were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO₂. At the indicated times, the cells were washed 3 times with ice-cold 0.154 M NaCl and harvested by adding ice-cold 0.05% trypsin-0.05% EDTA. The washing with saline has previously been shown to be an acceptable washing procedure for ellipticine drugs yielding a background of unspecific absorption lower than 5% (10). The cells were separated from the surrounding media by a variation of the filter system described by Conrad and Singer (11). In order to avoid suction, which has been shown to provoke a loss of cytoplasmic content, a 3-component filter was assembled consisting of a 25-mm diameter polycarbonate membrane, with an average pore diameter of 2 μm in the top (No. 110611; Nucleapore Corp.) a 25-mm diameter glass fiber paper GF/C (Whatman Ltd., Ferrières, France) in the middle, and a 4-mm thick pad of absorbent white cellulose in the bottom. The 3 filters were held together by a weighted stainless steel cylinder with a central core (diameter, 19 mm). After filtering of the cell suspensions, the top filters, which retain the cells, were dried and transferred to scintillation bottles, scintillation liquid (7 ml, Ready-Solv MP; Beckman) was added, and the radioactivity determined with a Beckman liquid scintillation spectrometer. All experiments were done 2 times in triplicate.

Efflux Kinetics. The cells were plated as described earlier and incubated for 4 h in MEM with 8% FCS containing AA-NMHE (5 nmol/ml). They were then washed 3 times with ice-cold 0.154 M NaCl and
reincubated in drug-free medium. Since a large fraction of drug can escape in the first few minutes (12) the time for change of medium was kept under 30 s in all cases. At the indicated times, the amount of drug still associated with the cells was determined as described above.

Cell Size Determination. The average cell size was determined by flow cytometry analysis, carried out with a Cytograf (Model 6300) coupled to a distribution analyzer (Model 2100; Biophysics System, Inc.). As standards, polystyrene microspheres (5.1 and 10.2 μm diameter, Nos. 269 and 270, respectively; Duke Scientific) were used.

Preparation of Nuclei. Nuclei were prepared by detergent treatment in isotonic medium (13). The nuclei remained attached to the support through cytoskeletal elements and studies on drug accumulation were performed by incubating the nuclei with AA-NMHE (5 nmol/ml) for 4 h followed by washing and harvesting as described for whole cells. For the nuclei a 25-mm diameter polycarbonate membrane with an average pore diameter of 0.2 μm (No. 110606; Nuclepore) was used.

Fluorescence Microscopy. Two days prior to the experiments 3 x 10^6 cells were planted on a glass lamella (34 x 34 mm) in a 6-cm diameter Petri dish (Nuclon) containing 7 ml of growth medium. After transfer of the lamellas to culture observation chambers (5.3 cm^2; 2 ml) the cells were incubated with AA-NMHE (5 nmol/ml) at 37°C for the indicated times. Alternatively, cells were planted on glass lamellas 2 days prior to the experiments, and nuclei were prepared by detergent treatment as described above. The nuclei were then incubated with growth media containing AA-NMHE (5 nmol/ml) for the indicated times. Initial experiments were carried out on a Zeiss Photomicroscope III fitted with a high-pressure mercury lamp (HBO 200 W) and the BG 12 filter combination (λ exc = 436 nm maximum; λ em > 520 nm). Photomicrographs were taken on Ilford film (HP5, 400 ASA; developed at 1600 ASA) with an exposure time of less than 30 s. Subsequent experiments were carried out with a Leitz Orthoplan microscope fitted with the Ploemopak 2.1 fluorescence system (λ exc = 300-400 nm; λ em > 450 nm). To process the image, a silicon intensified target (SIT) video camera (Bosh) was interfaced with a Magiscan 2 (Nachet) automated image analysis system. Neutral density filters were used to keep excitation intensities within the range of the digitizing system. The transfer of the image observed through the microscope to the memory take place within 50 ms. The reconstituted image then appears on the videoscreen; photos were taken of this image with a Polaroid camera (CV-5 88-49) using Polaroid 665 film (ASA 80).

Cytotoxicity. The in vitro colony formation technique was used to determine reproductive cell death. Cells designated for colony formation were counted, and about 1000 cells were replated in 60-mm sterile Petri dishes and incubated overnight to allow attachment of cells to the plate surface. The cells were treated with MEM with 8% FCS containing AA-NMHE for 4 h followed by aspiration of the medium. The plates were then washed with 0.154 M NaCl, and incubated at 37°C for 8 days after addition of 5 ml of conditioned medium. Alternatively the cells were incubated for 8 days in the presence of drug-containing conditioned medium. The colonies were then fixed with 100% methanol, stained with 0.5% crystal violet, and examined under a stereomicroscope. Colonies of 50 or more cells were considered to originate from viable cells. The mean ± SD of triplicate samples was determined for each drug concentration. The data were analyzed by plotting the log of the survival fraction (number of colonies in drug-treated plates per number of colonies in controls) versus the drug concentration and the drug concentration required to reduce colony formation by 63% on the exponential part of the survival curve was calculated. The plating efficiency of control cultures ranged from 20-30% in these experiments.

RESULTS

Drug Uptake as a Function of Concentration. Reciprocal plots of the rate of drug uptake as a function of drug concentration for NMHE, Ala-, and Ile-NMHE are depicted in Fig. 2. The amount of drug taken up in 10 min is directly proportional to drug concentration over a range of 0.5-40 μM, which suggests that uptake is nonsaturable and probably not carrier mediated. All the AA-NMHE conjugates show similar initial uptake as illustrated by Ala- and Ile-NMHE.

Drug Accumulation over Time. Drug accumulation as a function of time is shown in Fig. 3. The results show that drug uptake reaches a plateau after approximately 4 h of incubation for all drugs except Gly- and Ala-NMHE where drug uptake

![Fig. 1](image1.png)

**Fig. 1. Structure of amino acid-NMHE conjugates.** The numbering of atoms at positions 9 and 10 are indicated. R = H, Gly-NMHE; R = CH₂, Ala-NMHE; R = CH₂CH₂, Aba-NMHE; R = CH (CH₃)₂, Val-NMHE; R = CH₂(CH₂)₂Leu-NMHE; R = CH(CH₃)CH2CH₃, Ile-NMHE. The counter ion is acetate for all the conjugates.
increases throughout the entire 6-h experimental period. Sodium azide (10 mM) has no effect on the uptake of any of these drugs.

Drug accumulation by isolated nuclei reaches a plateau after about 1 h incubation. The drug uptake after 4 h uptake is shown in Table 1. The uptake of Gly-NMHE, Ala-NMHE, Aba-NMHE, and Val-NMHE is comparable to that which is observed for whole cells at this time (Fig. 3). In contrast, the nuclear uptake of NMHE by isolated nuclei far exceeds that observed for whole cells whereas the reverse is true for Ile-NMHE and Leu-NMHE.

In order to determine the intracellular drug concentration, the size of the cells was determined by flow cytometry. After trypsinization, the average cell diameter was close to 9 μm. Based on this value and the assumption that the trypsinized cells are spherical, it was calculated that after 4 h of incubation the intracellular concentration of AA-NMHE ranged from 2.5–3.6 mM which represents an over-concentration of approximately 500–720-fold compared to the external medium. In comparison, the intracellular concentration of NMHE was close to 0.5 mM or approximately 100 times the concentration of the external medium.

Drug Efflux. Release of AA-NMHE as a function of time following a 4-h incubation period is shown in Fig. 4. The efflux kinetics is similar for all drugs in that a rapid loss occurs the first 30 min after which the efflux slows down. After 3 h of efflux about 50% (range, 42–54%) of the AA-NMHE remains associated with the cells. In contrast, only 31% of the NMHE remains associated with the cells which indicates that a major part of this drug is loosely bound to the cells. The presence of sodium azide has no effect on drug efflux from preloaded cells.

Cytofluorescence Localization. The intracellular distribution of AA-NMHE was studied by fluorescence microscopy. Initial studies were performed with conventional fluorescence microscopy and resulted in a fluorescence pattern similar to the ones depicted in Fig. 5, A and C. Incubation with Gly-, Ala-, Aba-, or Val-NMHE resulted in predominantly nuclear fluorescence as shown in Fig. 5A while disperse fluorescence appeared in cells incubated with Ile- and Leu-NMHE as shown in Fig. 5C. NMHE is not fluorescent and therefore cannot be examined. In order to study the cellular distribution of AA-NMHE at a concentration below 5 μM we decided to detect the fluorescence through a SIT camera interfaced with an automated system which is more sensitive. In addition, the automated system enables us to obtain an image after less than 1 s exposure of the cells to fluorescent light. Using this system, we observed bright greenish fluorescence in cytoplasmic granules of various size as shown in Fig. 5, B and D.

The cytofluorescence was concentrated around the nucleus as seen most clearly at low drug concentrations (Fig. 5E).
Neither cytoplasmic membranes nor other cell components were fluorescent, whereas the chromosomes of dead cells were intensively fluorescent. Continued UV resulted in fluorescence translocation generating the same cytofluorescence pattern as originally observed by conventional fluorescence microscopy (Fig. 5, A and C). Once light-induced drug translocation had taken place it remained localized in the new cell compartments for at least 12 h.

Isolated nuclei displayed clear fluorescence at concentrations as low as 0.1 μM (Fig. 5F). This together with the translocation indicates that the lack of intranuclear fluorescence is not likely to result from fluorescence quenching in the nuclei.

Cytotoxicity of AA-NHME. The cytotoxicity of AA-NMHE conjugates was examined by the colony-forming assay following either 4 h or 8 days drug exposure. The survival curves of AA-NMHE- or NMHE-treated cells showed a monophasic dose-dependent exponential decrease. Table 2 shows the mean lethal dose which was calculated from the exponential survival curve as the dose necessary to reduce survival by 63%.

**DISCUSSION**

Our results show that the uptake of both NMHE and the AA-NMHE conjugates is rapid, directly proportional to drug concentration, and is unaffected by the metabolic inhibitor sodium azide. These results are consistent with a nonsaturable process, most likely diffusion. In addition, these drugs may be influenced by the electric membrane potential due to their positive charge as shown to be the case for 2-N-methyl-ellipticinium (14). The intracellular concentration of NMHE by 4 h, when a plateau is reached exceeds the external drug concentration by about 100-fold; this suggests that the toxicity of NMHE is not as previously assumed limited by its transport across tumor cell membranes. Conjugation of NMHE with aliphatic amino acids increased both the initial uptake and the drug accumulation with time. It is likely that the similarities in uptake observed at 10 min for the AA-NMHE conjugates are a reflection of similarities in membrane binding and permeability. The accumulation over time is influenced by additional factors including uptake, efflux, and binding to cellular storage sites. It is interesting that the uptake for all the aliphatic amino acid conjugates is about the same, independent of the character of the hydrophobic moiety. The cellular uptake of the AA-NMHE conjugates by 4 h represents an overconcentration of up to 700-fold the concentration of the external medium or about 7 times more than observed for NMHE. This result suggests that the AA-NMHE conjugates bind with higher affinity to the binding sites than does NMHE. Alternatively, the AA-NMHE might have additional binding sites. A similar overconcentration of drug has been described for other agents such as actinomycin D (15), daunomycin (16), and Adriamycin (17) and indicates that once inside the cells these drugs bind to structures with high drug affinity and/or numerous binding sites.

The cytofluorescence studies show that most if not all of the AA-NMHE is located in the cytoplasm of living cells. The granular cytoplasmic fluorescence is probably due to AA-NMHE captured by lysosomes as previously demonstrated for Adriamycin (18). This finding is also supported by the observation that a number of cationic dyes are concentrated in subcellular organelles such as mitochondria and lysosomes as a consequence of the transmembrane potential (19, 20); together, these findings suggest but do not prove that the AA-NMHE conjugates have their effects on the cytoplasm. We cannot exclude the possibility, however, that a small fraction of drug, physiologically important, might be located in the nucleus.

The cytoplasmic fluorescence is readily translocated resulting in either predominantly nuclear fluorescence (Gly, Ala-, Aba-, and Val-NMHE) or diffuse fluorescence throughout the cell (Leu- and Ile-NMHE). This is accompanied by the disappearance of the granular cytoplasmic structures. The reason for the translocation is not known but might be due to a light and/or temperature effect. Once translocated, the nuclear fluorescence remains stable for at least 12 h.

The results of the cytofluorescence studies illustrate some practical points to be considered in drug localization studies. Such studies are usually based on either cytofluorescence microscopy or subcellular fractionation. We here show that it is possible for drug translocation to take place within a few seconds. This is too fast to be observed with conventional microscopy and can only be appreciated by using automated systems that enable us to “freeze” the image after less than 1 s exposure to fluorescent light. In addition, the results show that NP40, a detergent commonly used for subcellular fractionation, is able to induce changes in drug cellular distribution, the most obvious change being the drug translocation to the nucleus. Similar findings have been described for 2-N-methyl-ellipticinium in Chinese hamster cells (10); together, these findings warrant extreme caution in the design of drug distribution studies.

Our findings could also have some clinical relevance. Recent studies of Adriamycin accumulation in drug-resistant colon cancer cells suggest that these cells might owe their resistance to their ability to exclude Adriamycin from the nucleus (21). Experimental manipulations resulting in restoration of nuclear fluorescence also restore the cytotoxicity (21). Although speculative at this point, it is possible that the cellular location and thereby the cytotoxicity of some anticancer drugs could be modified similarly by clinical treatments such as hyperthermia and/or X-ray irradiation.

No obvious relationship was detected between drug accumulation and cytotoxicity as determined by the colony forming assay. All the AA-NMHE conjugates are less toxic than the parent compound after 4 h drug exposure in spite of their improved intracellular accumulation. This effect might be explained by the inability of the AA-NMHE conjugates to undergo oxidative bioactivation to phenoxyl radicals which has been described to be part of the cytotoxic action of NMHE (22). Alternatively, the cellular distribution of NMHE could be different from that of the AA-NMHE conjugates. The Ile- and Leu-NMHE conjugates exhibit the highest cytotoxicity; both of these drugs are effective in the ng range after 8 days of drug exposure. The cytotoxic effect of the Ile-NMHE conjugate is
accumulative with time; increase of drug exposure from 4 h to 8 days results in 70 times augmentation of the cytotoxicity. In contrast, cytotoxicity only increases 9–17 times for the other drugs; this suggests that the mechanism of action of the Ile-NMHE is different from the rest. Studies on the cytotoxic action in vivo are now in progress in our laboratory and should provide a better knowledge of the clinical potential of these drugs.

In conclusion, our results show that the presence of a hydrophobic moiety at the C-10 position of NMHE results in increased intracellular accumulation; however, except for the Ile-NMHE conjugates, this effect is associated with a decrease in cytotoxicity which might be due to loss of the phenolic character of the NMHE moiety; therefore, attempts to linken NMHE to drugs. This suggests that the mechanism of action of the Ile-drugs only increases 9–17 times for the other drugs; this suggests that the mechanism of action of the Ile-drugs is different from the rest. Studies on the cytotoxic action in vivo are now in progress in our laboratory and should provide a better knowledge of the clinical potential of these drugs.

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