Kinetics of cyclocreatine and Na\textsuperscript{+} cotransport in human breast cancer cells: mechanism of activity

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\textsuperscript{1}Department of Biological Regulation, Weizmann Institute of Science, Rehovot 76100, Israel; \textsuperscript{2}Department of Chemistry, University of Texas at Dallas, Richardson, Texas 75083-0688; and \textsuperscript{3}Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6089

Maril, Nimrod, Hadassa Degani, Edna Rushkin, A. Dean Sherry, and Mildred Cohn. Kinetics of cyclocreatine and Na\textsuperscript{+} cotransport in human breast cancer cells: mechanism of activity. Am. J. Physiol. 277 (Cell Physiol. 46): C708–C716, 1999.—The growth-inhibitory effect of cyclocreatine (CCr) and the kinetics of CCr and Na\textsuperscript{+} cotransport were investigated in MCF7 human breast cancer cells and its adriamycin-resistant subline with use of $^{31}$P- and $^{23}$Na-NMR spectroscopy. The growth-inhibitory effect in the resistant line occurred at a lower CCr concentration and was more pronounced than in the wild-type line. This correlated with an $\sim$10-fold higher affinity of CCr to the transporter in the resistant line. The passive diffusion coefficient of CCr was also higher in the resistant line by three- to fourfold. The transport of CCr was accompanied by a rapid increase in intracellular Na\textsuperscript{+}. This increase was found to depend on the rate of CCr transport and varied differently with CCr concentration in the two cell lines. It is proposed that the cotransport of CCr and Na\textsuperscript{+} followed by increased Na\textsuperscript{+} concentration, together with the accumulation of the highly charged phosphocyclocreatine, are responsible for cell swelling and death.

phosphorus-31 nuclear magnetic resonance; sodium-23 nuclear magnetic resonance; cell perfusion

cyclocreatine [1-carboxymethyl-2-iminoimidazolidine (CCr)], a creatine (Cr) analog, has been shown to act as an anticancer agent in a large range of cell culture systems and in tumors implanted subcutaneously in rats and mice (25, 31, 34, 41, 47). The mechanism of cell death by CCr in C\textsubscript{6} glioma cells appeared to be related to the accumulation of phosphocyclocreatine (PCCr) and cell swelling (41, 42). However, the detailed mechanism by which CCr enhances cell death is unknown.

CCr and Cr are transported into the cells and are rapidly phosphorylated by the creatine kinase (CK) reaction to form PCCr and phosphocreatine (PCr), respectively, according to the following scheme

$$
CCr_e \xrightarrow{k_{ccr}} CCr_i + MgATP^2- \\
\xleftarrow{k_{pcr}} PCCr^2- + MgADP^- + H^+
$$

where CCr\textsubscript{i} is intracellular CCr, CCr\textsubscript{e} is CCr in the perfusion medium, $k_{ccr}$ is the apparent first-order rate constant of CCr transport, and $k_1$ and $k_{-1}$ are the forward and backward rate constants of the CK reaction, respectively (33). The CK reaction is a dead-end reaction and, in general, serves to regulate energy in the cell (for review see Ref. 52). Among all Cr analogs, CCr is the best substrate of the CK reaction by the $V_{max}/K_m$ criterion (where $V_{max}$ is maximal reaction velocity and $K_m$ is Michaelis-Menten constant) (3, 29, 33, 50, 54). The rate of ATP generation from PCCr is 160-fold slower than the rate of ATP generation from PCr, and the reverse reaction is 5-fold slower (3). This resulted in an $\sim$30-fold higher equilibrium constant of the CK reaction when CCr served as a substrate of CK (scheme 1). Thus, when rats are fed CCr in the diet, at equilibrium 98% of the total Cr in various tissues is present as PCCr (50). In the body, Cr is predominantly synthesized in the pancreas, liver, and kidney and is then exported to the circulating blood and transported into the cells (48, 49). The transport has been well studied, and recently the human Cr transporter has been cloned (36) and characterized after expression in Xenopus oocytes (12). Although there is no universal detailed mechanism proposed by the various investigators, all have revealed an Na\textsuperscript{+}-dependent component (12, 13, 27, 28, 35). In cell culture studies, two saturable processes or a combination of a saturable transport component and a diffusion component were identified (13, 27, 28, 35). In $^{31}$P-NMR studies of Cr uptake in liver expressing CK, a pH-dependent saturable Michaelis-Menten type of kinetics was described (32). By monitoring PCCr with use of $^{31}$P-NMR, active (Michaelis-Menten) and diffusion mechanisms were found to contribute to the transport of CCr in ex vivo studies of uterine tissue (45). In $^{31}$P-NMR studies of C\textsubscript{6} glioma cells, the active component of CCr transport was characterized and its Na\textsuperscript{+} dependence was demonstrated (41).

$^{31}$P-NMR detects not only the PCr, but also other phosphorus-containing metabolites. Of special significance is the level of nucleoside triphosphates (NTPs) and internal $P_i$, which reflect the energy state of the cells (15). $^{23}$Na-NMR of perfused cells detects the overlapping signals of the intra- and extracellular Na\textsuperscript{+}. However, the use of an anionic relaxation or frequency shift reagent results in the removal of this degeneracy (14, 19, 40). The anionic chelate complexes of paramagnetic lanthanides, particularly Dy\textsuperscript{3+} and Tm\textsuperscript{3+}, on binding to the Na\textsuperscript{+}, cause a resonance hyperfine shift of the $^{23}$Na\textsuperscript{+} signal. Because the anionic paramagnetic

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shift reagents do not penetrate the cell membrane within the time scale of NMR measurements (1–2 days), only the extracellular Na\(^+\) ions experience the resonance shift. Thulium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) (TmDOTP\(^5^-\)) has been shown to be stable in blood and was successfully used as a shift reagent to detect the intracellular Na\(^+\) in animal experiments (4, 5, 30, 43). In early in vivo studies with this shift reagent (1), it was toxic at high levels; in later studies (4, 5, 43), with proper anesthesia, it was well tolerated by rats. At the concentration used here (10 mM) for cell culture studies, TmDOTP\(^5^-\) did not affect cell energetics (i.e., NTP concentration) and appeared to be nontoxic.

The goal of the present studies was to elucidate the mode of action of CCR as an antitumor agent. We investigated and compared the effect of CCR on the growth rate and the morphology of MCF7 wild-type (WT) and the adriamycin-resistant clone MCF7 ADR\(^\text{R}\) human breast cancer cell lines (16). \(^{31}\)P-NMR and \(^{23}\)Na-NMR were applied to determine noninvasively the kinetics of CCR and Na\(^+\) cotransport, respectively, in these cells. The higher sensitivity of the MCF7 ADR\(^\text{R}\) clone to CCR than that of the WT clone was correlated with the kinetic parameters of the cotransport of CCR and Na\(^+\) in the two cell lines.

MATERIALS AND METHODS

Cell culture. MCF7 and MCF7 ADR\(^\text{R}\) human breast cancer cells were kindly supplied by Dr. Lippman (National Institutes of Health, 1987 and 1995, respectively). MCF7 cells were routinely cultured in DMEM supplemented with 6% FCS (Biological Industries, Beit-Haemek, Israel) and 0.1% combined antibiotics (Bio-Lab, Jerusalem, Israel) containing penicillin (200,000 U/ml), streptomycin (200,000 µg/ml), and neomycin (10,000 µg/ml). MCF7 ADR\(^\text{R}\) cells were cultured routinely with a 1:1 mixture of minimum essential medium and F-12 medium supplemented with 10% FCS, 0.1% combined antibiotics, and additional glucose (1 g/l), sodium bicarbonate (1 g/l), choline chloride (50 mg/l), and inositol (30 mg/l). The final growth medium contained a negligible amount (−22 µM) of Cr, which was present only in the serum (2).

In cell experiments with Cr (Sigma Chemical) or CCR [synthesized by the method of Wang (53)], stock solutions of medium containing each metabolite at 40 mM were used to reach a final concentration of 0.3–19 mM.

Growth studies. Cells were plated in 24-well dishes (Nunc) at a density of −1.5 × 10⁵ cells/well in 1 ml of culture medium and placed in a 37°C, 5% CO\(_2\) humidified incubator. After 48 h, the medium was replaced by fresh medium containing various concentrations of CCR or Cr. Growth of cells was followed by cell counting with an inverted light microscope by use of a hemocytometer. Inhibition of cell growth was measured spectroscopically on the supernatant of sonicated cells. CK activity was measured spectrophotometrically at 340 nm with use of a coupled assay, as previously described (44). Protein was determined by the Bradford method (7). CK activity is given as the mean ± SE of three independent experiments, in units of micromoles per minute per milligram protein.

Morphological assessment of cell cultures. MCF7 and MCF7 ADR\(^\text{R}\) cells were cultured in petri dishes as described above. Two days after seeding, the standard growth medium was replaced every 48 h by fresh medium for the controls and, in addition, 20 mM CCR for the treated cells. Both cell cultures were examined at 24-h intervals with an inverted light microscope to monitor changes in the diameter of the cells and to calculate the mean area (n = 20) with the assumption of a circular shape. The change in area of the cells during treatment with CCR is reported as the ratio of the mean area before to that after treatment plus or minus the uncertainty.

Cell cultures during NMR experiments. For NMR studies, cells (MCF7 and MCF7 ADR\(^\text{R}\)) were cultured on agarose-polyacrylon beads (250–500 µm) or on Biosilon polystyrene beads (160–300 µm, Nunc) with the growth medium described above by following procedures described previously (37). The cells grown on beads (2–2.5 ml) were placed in a 10-mm sterile NMR tube and perfused with oxygenated medium at 36 ± 1°C, as described previously (15). In the \(^{31}\)P-NMR studies the cells were perfused with DMEM containing 10% FCS and combined antibiotics. In the \(^{23}\)Na-NMR studies, cells were perfused with Na\(^+\)-free growth medium as described above containing the shift reagent NaH\(_2\)TmDOTP·3NaCl at 10 mM (8) and additional NaCl to reach a final concentration of 155 mM Na\(^+\).

NMR experimental parameters. NMR spectra were recorded with a Bruker AM-500 spectrometer with use of a multinuclei broad-band probe. \(^{31}\)P-NMR spectra were recorded at 202.5 MHz by acquiring 900 transients with 45° pulses, 59 µs between the pulse and the acquisition (DE), 2-s repetition time, and 0.38-s acquisition time, with a continuous composite pulse proton decoupling of −1 W. The chemical shift of \(^{31}\)P signals was referenced to a-NTp at −10.03 ppm. Concentrations were calculated from the integrated areas of the corresponding signals, with the integrated area of medium P\(_i\) (1 mM) used as reference and correction for saturation effects. Analysis of the \(^{31}\)P spectra was performed with a standard Bruker software package, XWIN-NMR, with application of integration and line shape-fitting modes.

\(^{23}\)Na-NMR spectra were recorded at 132 MHz by acquiring 2,000 transients with 90° pulses, DE of 86 µs, 0.47-s repetition time, and 0.27-s acquisition time. Concentration of the intracellular Na\(^+\) was calculated from the integrated area of its signal referenced to the integrated area of the medium Na\(^+\) signal (155 mM, 100% NMR visibility) with the assumption of 40% NMR visibility of the intracellular Na\(^+\). In most cells the intracellular Na\(^+\) signal is only 40% visible by NMR compared with the extracellular Na\(^+\) signal, which is 100% visible (6). When DE is very short, a full visibility of the intracellular Na\(^+\) might be regained. However, for the DE value applied here, the assumption of 40% visibility is justified. The energy state of the cells and the extent of PCCr accumulation in the \(^{23}\)Na experiments were characterized by recording \(^{31}\)P spectra at the beginning and end of the experiments with the cells perfused with the standard growth medium in the absence of the shift reagent. The integrated areas of the \(^{23}\)Na signals were obtained with a standard Bruker software package, XWIN-NMR.

RESULTS

Cell growth and CK activity. Studies of the growth of MCF7 and MCF7 ADR\(^\text{R}\) cells in the presence of various concentrations of CCR indicated a dose-dependent growth inhibition by this drug (Fig. 1, A and B). The sensitivity to the drug was different in each cell line. In the MCF 7 line, cells continued to grow in the presence

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of CCr, but at a slower rate than untreated cells (Fig. 1A). For example, after 5 days in the presence of 20 mM CCr, the number of cells relative to day 0 increased by 28%, but the percent reduction of cell number from control was 61%. On the other hand, in MCF7 AdrR cells, CCr markedly inhibited growth (Fig. 1B); after 5 days in the presence of 20 mM CCr, the number of cells relative to day 0 actually decreased by 23% and the percent reduction from control was 93%. In a similar study with Cr in MCF7 AdrR cells, contrary to the behavior of CCr, only a marginal inhibition of growth was observed (Fig. 1C). At a very high concentration of Cr (40 mM) and after 7 days of treatment, reduction of MCF7 AdrR cell number relative to control was 52%.

The activity of CK was 0.026 ± 0.009 and 0.069 ± 0.032 μmol·min⁻¹·mg protein⁻¹ in MCF7 cells (n = 3) and in the resistant cell line (n = 3), respectively. Thus the mean CK activity was almost threefold higher in the resistant than in the WT cells.

Cell swelling. MCF7 AdrR cells grown as a monolayer in the presence of 20 mM CCr exhibited distinct morphological changes and marked cell swelling (Fig. 2). After 6 days of treatment the mean cell area increased by 3.0 ± 0.2-fold (ratio of means ± uncertainty) compared with control cells. The WT cells in the presence of the same concentration of CCr (20 mM) exhibited less swelling, and after 6 days of treatment their mean area increased by 1.5 ± 0.5-fold (ratio of means ± uncertainty). At this concentration the transport of CCr is dominated by a diffusion mechanism, for which the rate is about fourfold higher in the resistant line than in the WT (see below).

CCr transport measured by 31P-NMR. 31P spectra of both cell lines exhibited signals of phosphorus in soluble metabolites that are present at millimolar concentrations (Figs. 3B and 4B), including NTPs, PCr, and Pi (intra- and extracellular), all associated with cell energetics (11). The PCr signal was more intense in MCF7 AdrR than in WT cells (Figs. 3B and 4B). In addition, signals due to phosphocholine, phosphoethanolamine, and uridine diphosphosugars were detected. Glycerophosphoethanolamine and glycerophosphocholine were present at low concentrations in WT cells (Fig. 3) and below detection in the resistant clone (Fig. 4).

After addition of CCr to the perfusion medium, a distinct signal from PCCr (−2.34 ppm) close to that of
PCr appeared in the 31P spectrum (Figs. 3A and 4A). Initially, it was possible to resolve PCr and PCCr signals. During this period the PCr signal intensity did not change. After several hours of perfusion with CCr, as PCCr became predominant, the two signals could not be resolved. However, with the assumption that PCr is constant, the increase of the combined signal of PCr and PCCr reflected PCCr accumulation.

The mechanism and kinetic parameters of CCr transport into MCF7 and MCF7 AdrR cells were determined by sequentially increasing CCr concentration in the perfusion medium and monitoring PCCr with 31P-NMR. The rate of increase in the PCCr signal is limited by the rate of transport of CCr or by the rate of the phosphorylation through the CK reaction. The following analysis of previous kinetic data supports the assumption that transport of CCr is the rate-limiting step. The CK-catalyzed rate of PCCr synthesis from Cr in T47D human breast cancer cells, which demonstrate a CK activity and ATP-to-PCr ratio similar to MCF7 AdrR cells, was 0.2 mM/s (38). Inasmuch as the rate of PCCr synthesis is fivefold slower than the rate of CCr synthesis (3), the estimated CK flux to form PCCr in MCF7 AdrR cells is ~0.04 mM/s. Similarly, a threefold lower flux is estimated for the CK reaction in the WT cells on the basis of their lower CK activity (see above). The following analysis of previous kinetic data supports the assumption that transport of CCr is the rate-limiting step. The CK-catalyzed rate of PCr synthesis from Cr in T47D human breast cancer cells, which demonstrate a CK activity and ATP-to-PCr ratio similar to MCF7 AdrR cells, was 0.2 mM/s (38). Inasmuch as the rate of PCCr synthesis is fivefold slower than the rate of CCr synthesis (3), the estimated CK flux to form PCCr in MCF7 AdrR cells is ~0.04 mM/s. Similarly, a threefold lower flux is estimated for the CK reaction in the WT cells on the basis of their lower CK activity (see above). The Michaelis-Menten $V_{\text{max}}$ derived from the rates of PCCr increase ($Q_{\text{eq}}$) in both cell lines was on the order of 3 fmol·cell$^{-1}$·h$^{-1}$ (Table 1), which is equivalent to ~0.0005 mM/s (average cell volume of 1,500 µm$^3$). This rate is more than one order of magnitude slower than the above-estimated rates of PCCr synthesis by CK and can be attributed only to the transport. Therefore, the rate of PCCr increase served to measure CCr transport by applying the zero trans method (46).

The initial change with time in the intensity of the PCCr signal was linear. The apparent initial rate ($V_{\text{app}}$) as a function of CCr concentration was best fitted to Eq. 2 with two mechanisms of transport: 1) saturable, Michaelis-Menten-like active transport and 2) passive diffusion (Fig. 5)

$$V_{\text{app}} = \frac{d[PCCr]}{dt} = \frac{V_{\text{max}} \times S}{K_m + S} + K_d \times S \quad (2)$$

where [PCCr] is PCCr concentration, S is CCr concentration in the medium, $V_{\text{max}}$ is the maximum velocity, $K_m$ is the Michaelis Menten constant, and $K_d$ is the passive diffusion constant. The resultant kinetic parameters are summarized in Table 1. The results clearly demonstrated that the $K_m$ of active transport in the resistant line was an order of magnitude lower than that in the WT cells, whereas $V_{\text{max}}$ was similar. In addition, $K_d$ was fourfold higher in the resistant line.

CCr and Cr effect on intracellular Na$^+$ content. The transport of Na$^+$ concomitant with the transport of CCr was monitored by changes in the intracellular Na$^+$ concentration with 23Na-NMR. 23Na spectra of WT and

Table 1. Kinetic parameters of CCr transport

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$K_m$, mM</th>
<th>$V_{\text{max}}$, fmol·cell$^{-1}$·h$^{-1}$</th>
<th>$K_d$, fmol·cell$^{-1}$·h$^{-1}$·mM$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>4.41 ± 0.8</td>
<td>3.28 ± 0.65</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>MCF7 AdrR</td>
<td>0.44 ± 0.08</td>
<td>3.03 ± 0.61</td>
<td>0.95 ± 0.19</td>
</tr>
</tbody>
</table>

Values are means ± SE. $K_m$, Michaelis-Menten constant; $V_{\text{max}}$, maximal reaction velocity; $K_d$, diffusion coefficient; CCr, cyclocreatine.
resistant cells perfused with medium containing 10 mM TmDOTP$^{5-}$ as a shift reagent revealed two separate signals (Fig. 6). The extracellular Na$^+$ signal was shifted downfield by $-13$ ppm from the intracellular Na$^+$ signal, arbitrarily assigned to 0 ppm, making it possible to detect and quantitate the signal of each Na$^+$ compartment separately. The shift of the extracellular Na$^+$ signal decreased with time in parallel to the decrease in the medium pH (Fig. 6), as previously reported (23). After 15 h the shift of the extracellular Na$^+$ was $-10$ ppm (Fig. 6A); however, this shift was still large enough for complete resolution of the two signals. To examine the stability of the steady-state Na$^+$ level, cells were initially perfused for 9 h in the presence of 10 mM TmDOTP$^{5-}$. During this period there was no change in the Na$^+$ content, confirming that neither the NMR conditions nor the presence of 10 mM TmDOTP$^{5-}$ in the perfusion medium affected the intracellular Na$^+$ content (Fig. 7A).

Addition of CCr to the perfusion medium induced an increase in the intracellular Na$^+$ signal (Fig. 6). The time course of the rapid increase of the intracellular Na$^+$ content in MCF7 Adr$^R$ cells perfused with 10 mM CCr is shown in Fig. 7B with a rate of $3.7 \pm 0.7$ fmol·cell$^{-1}$·h$^{-1}$. At the beginning of the experiment the intracellular Na$^+$ content was 77 fmol/cell. Seventeen hours after CCr addition, the intracellular Na$^+$ content was 155 fmol/cell. Then the level of the intracellular signal started to decrease (Fig. 7), paralleling cell
death, as confirmed by $^{31}$P spectra obtained at the end of experiments. When Cr was added to the perfusion medium of MCF7 AdrR cells, a very slow increase in the intracellular Na$^+$ (relative to the increase in the presence of CCr) of $1.5 \pm 0.3$ fmol·cell$^{-1}$·h$^{-1}$ occurred. After 7 h the initial Na$^+$ level (78 fmol/cell) reached a steady state of 90 fmol/cell, which persisted until the end of the experiment (Fig. 7A).

The rates of intracellular Na$^+$ accumulation and of CCr transport have been compared to determine whether a correlation exists between them. The rates of CCr transport via the active transport mechanism in the two cell lines were calculated on the basis of the $V_{max}$ and $K_m$ values found in $^{31}$P-NMR transport experiments (Table 2). In the resistant line at 1 and 10 mM CCr, the transport rates are close to the saturation rate. However, in the WT line, the rate at 10 mM CCr is close to saturation, whereas at 1 mM the transport rate is ~3.5-fold slower. Therefore, we have chosen to measure the intracellular Na$^+$ accumulation rates in both cell lines at 1 and 10 mM CCr (Fig. 8) and determined the ratio of these rates for both cell lines (Table 3). This ratio is independent of the assumption of 40% Na$^+$ visibility in the intracellular compartment and of the experimental variability in the initial Na$^+$ concentration (65–100 fmol/cell). The ratios of intracellular Na$^+$ accumulation rates in the presence of 10 mM to those in the presence of 1 mM CCr were similar to the ratios of the transport rates of CCr through the active transport component at 10 mM to those at 1 mM CCr (Tables 2 and 3).

**DISCUSSION**

The mechanism by which CCr could exert its anticancer activity was investigated in two lines of MCF7 human breast cancer cells: the WT cell and its adriamycin-resistant subline. CCr induced a time- and dose-dependent inhibition of growth in the two cell lines. The resistant cells exhibited a much higher sensitivity to CCr with a distinct cytotoxic effect.

In general, cancer cells with resistance induced by one therapeutic agent become resistant to other drugs or are unaffected by them; rarely do they become more sensitive to structurally unrelated agents, as in the case of CCr with MCF7 AdrR cells presented in this report. A similar phenomenon of increased toxicity in MCF7 AdrR cells has been observed with 2-deoxyglucose (2-DG) (20, 21). In that study the greater toxicity

**Table 2. CCr transport rates by saturable mechanism**

<table>
<thead>
<tr>
<th>CCr Concentration, mM</th>
<th>Calculated Rate, fmol·cell$^{-1}$·h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF7 cells</td>
</tr>
<tr>
<td>1</td>
<td>0.6 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>2.27 ± 0.45</td>
</tr>
<tr>
<td>Rate at 1 mM/rate at 10 mM</td>
<td>3.78 ± 0.75</td>
</tr>
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Values are means ± SE calculated from the $V_{max}$ and $K_m$ values in Table 1.

**Fig. 8. Na$^+$ accumulation in MCF7 (A and C) and MCF7 AdrR cells (B and D) in presence of 10 mM CCr (A and B) or 1 mM CCr (C and D). Cells were cultured and perfused in NMR spectrometer. Na$^+$ content was determined from integrated area of intracellular $^{23}$Na-NMR signal. Na$^+$, intracellular Na$^+$.**
Table 3. Rates of intracellular Na\(^+\) accumulation in presence of CCr

<table>
<thead>
<tr>
<th>CCr Concentration, mM</th>
<th>Na(^+) Accumulation Rate, fmol·cell(^-1)·h(^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7 cells</td>
<td>MCF7 Adr(^R) cells</td>
</tr>
<tr>
<td>1</td>
<td>2.87 ± 0.57</td>
</tr>
<tr>
<td>10</td>
<td>2.08 ± 0.38</td>
</tr>
<tr>
<td>Rate at 1 mM/rate at 10 mM</td>
<td>0.63 ± 0.17</td>
</tr>
<tr>
<td>6</td>
<td>0.23 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE calculated from linear fit of Na\(^+\) accumulation data presented in Fig. 8.

in the resistant cells was ascribed to many factors, especially the faster depletion of ATP to form phospho-2-deoxyglucose (P-DG). The increased rate of accumulation of P-DG in the resistant cells relative to WT cells as monitored by \(^{31}\)P-NMR was ascribed to faster phosphorylation rather than faster transport into the cell. Another example of an anticancer agent to which resistant cells are more sensitive than the parental line is ardeemins; no mechanism was suggested (9). In the present study we attempted to elucidate the cause of the toxic activity of CCr by pinpointing the source of the quantitative differences between the WT and the resistant cells.

The accumulation of PCCr in the cell is governed by two processes: transport of CCr into the cell and its reversible phosphorylation by ATP catalyzed by CK. The essential presence of CK for the cytotoxic activity of CCr was demonstrated previously in a study of a large number of cancer cell lines (25). However, when CK reaches high enough activity, the phosphorylation is no longer rate limiting, and the transport capacity may become dominant in determining CCr cytotoxicity. The CK level in both cell lines, as noted in results, is sufficiently high not to be rate limiting. Furthermore, the two- to threefold higher CK activity in the resistant line cannot explain the markedly higher sensitivity to CCr of the resistant cells. This was further supported by measurements of CK activity and cell growth inhibition by CCr in T47D cells (not shown). Although in these cells the CK activity (0.058 ± 0.031 µmol·min\(^{-1}\)·mg protein\(^{-1}\)) was close to that of the MCF7 Adr\(^R\) clone, their sensitivity to CCr was very low, even less than that of MCF7 WT cells.

In contrast to the ~2.5-fold difference in CK activity, we found that the K\(_m\) of active transport of CCr in the resistant clone is 10-fold lower than that in the WT cells. With a 10-fold higher affinity of the transporter for its substrate (CCr) in the resistant clone, a higher sensitivity of these cells is obtained at concentrations of CCr below the K\(_m\) of active transport. The kinetic studies carried out with \(^{31}\)P-NMR of perfused cells also yielded the V\(_{max}\) of CCr transport and the coefficient of the diffusion component of the transport (Table 1). At concentrations of CCr high above the K\(_m\) (>10 mM), the active transport proceeds at a V\(_{max}\) that is similar for both cell lines. However, at these concentrations the fourfold higher diffusion coefficient in the resistant clone may also contribute to the difference in the sensitivity of the cells to CCr. The higher diffusion rate leads to a more rapid increase in intracellular CCr, which is rapidly converted to PCCr. Because the intracellular equilibrium concentration ratio of PCCr to CCr is high (3, 29, 54), CCr continues to diffuse into the cell, causing an accumulation of PCCr and an osmotic imbalance, which together with Na\(^+\) accumulation by the active transport component leads to cell swelling and death.

The active transport, for which K\(_m\) values were found to differ markedly between the two cell clones, has been previously shown to be Na\(^+\) dependent (13, 18, 27, 28, 35, 41, 45). Therefore, we have considered the role Na\(^+\) may play in the anticancer activity of CCr. In the initial studies the role of Na\(^+\) was indirectly characterized by measuring changes in the rate of Cr or CCr transport at various Na\(^+\) concentrations. The most direct method of evaluating the role of Na\(^+\) is to follow the intra- and extracellular Na\(^+\) by \(^{23}\)Na-NMR simultaneously with PCCr by \(^{31}\)P-NMR during the CCr transport process into MCF7 and MCF7 Adr\(^R\) cells.

In the presence of CCr in the perfusing medium, a linear intracellular accumulation of Na\(^+\) with different rates was observed in both cell lines. The agreement between the rates of CCr-induced intracellular Na\(^+\) accumulation, observed and anticipated from the respective kinetic parameters of CCr transport in the two cell lines, contributes strong additional evidence for a cotransport mechanism. A similar cotransport process was described for glucose (39) and for amino acid transport (10, 22).

In the resistant clone, because of the low K\(_m\) of CCr transport (0.4 mM) the rates of Na\(^+\) accumulation at 1 and 10 mM CCr were close. In the WT cells, where K\(_m\) was higher (4 mM), a marked difference in the rates of Na\(^+\) accumulation, at the two CCr concentrations, was measured. The similarity of the rates of Na\(^+\) accumulation at 10 mM in the two cell lines was consistent with the similarity of the V\(_{max}\) values. In the presence of Cr, a slow increase in the Na\(^+\) concentration to a steady-state level (90 fmol/cell) was observed, unlike the rapid intracellular accumulation of Na\(^+\) to much higher concentrations (160 fmol/cell) in the presence of CCr. This correlates with the sensitivity of the cells to CCr, and not to Cr, the natural substrate.

The Na\(^+\) accumulation could occur as a result of inhibition by a direct binding of CCr and/or PCCr to the Na\(^+\)-K\(^+\)-ATPase, as in the case of ouabain (26). Alternatively, the higher Na\(^+\) concentration, which accompanies CCr transport, due to the more favorable CK equilibrium constant, may saturate the Na\(^+\)-K\(^+\)-ATPase pumps so that the efflux becomes slower than the influx. Undoubtedly, a contributory mechanism is an indirect inhibition of Na\(^+\) efflux caused by the inability of the cells to pump out the Na\(^+\) for energetic reasons. ATP needed to operate the Na\(^+\) pump can be regenerated by the CK reaction from PCr by a direct functional coupling between the CK reaction and the Na\(^+\) pump (17, 24, 51). However, in the presence of PCCr, the regeneration of ATP is two orders of magni-
tude slower, and the energy supply may not be sufficient to fully activate the pump.

In summary, it is proposed that the impairment of cells in maintaining $Na^+$ homeostasis in the presence of $Cr$ (unlike $Cr$), leading to a net accumulation of $Na^+$ together with the accumulation of the highly charged PCCr, is responsible for cell swelling and death. The difference in sensitivity of various cell lines to $Cr$ may be ascribed to differences in their kinetic parameters of active cotransport of $Cr$ and $Na^+$.

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