Creatine and cyclocreatine treatment of human colon adenocarcinoma xenografts: $^{31}$P and $^1$H magnetic resonance spectroscopic studies

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Summary
Creatine (Cr) and cyclocreatine (cyCr) have been shown to inhibit the growth of a variety of human and murine tumours. The purpose of this study was to evaluate the anti-tumour effect of these molecules in relation to drug accumulation, energy metabolism, tumour water accumulation and toxicity. Nude mice carrying a human colon adenocarcinoma (LS174T) with a creatine kinase (CK) activity of 2.12 units mg$^{-1}$ protein were fed Cr (2.5% or 5%) or cyCr (0.025%, 0.1% or 0.5%) for 2 weeks and compared with controls fed standard diet. Cr concentrations of 2.5% and 5% significantly inhibited tumour growth, as did 0.1% and 0.5% cyCr. In vivo $^{31}$P magnetic resonance spectroscopy (MRS) after 2 weeks of treatment showed an increase in [phosphocreatine ($\text{PCr}$)+phosphocyclocreatine ($\text{PcyCr}$)]/nucleoside triphosphate (NTP) with increasing concentrations of dietary Cr and cyCr, without changes in absolute NTP contents. The antiproliferative effect of the substrates of CK was not related to energy deficiency but was associated with acidosis. Intratumoral substrate concentrations (measured by $^1$H-MRS) of 4.8 $\mu$mol g$^{-1}$ wet weight Cr (mice fed 2.5% Cr) and 6.2 $\mu$mol g$^{-1}$ cyCr (mice fed 0.1% cyCr) induced a similar decrease in growth rate, indicating that both substrates were equally potent in tumour growth inhibition. The best correlant of growth inhibition was the total Cr or (cyCr+Cr) concentrations in the tissue. In vivo, these agents did not induce excessive water accumulation and had no systemic effects on the mice (weight loss, hypoglycaemia) that may have caused growth inhibition.

Keywords: creatine; cyclocreatine; tumour xenograft; magnetic resonance spectroscopy; tumour growth

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adenocarcinoma implanted subdermally in nude mice. This tumour line has previously been used for extensive studies of tumour physiology and drug therapy, it has a relatively high CK activity and it is growth inhibited by Cr and cyCr. In view of the mechanisms presumed to contribute to the growth-inhibitory effects of these agents, we have evaluated: (1) intratumoral accumulation of Cr and cyCr delivered orally; (2) concentrations of the phosphorylated products of the CK substrates (PCr and PcyCr) and energy metabolic state of the tumours determined by in vivo 31P-MRS; (3) tumour water content and (4) systemic toxicity of these agents during a 2-week feeding period. These data were analysed with reference to CK activity and the isoenzyme distribution in the tumour.

MATERIALS AND METHODS

Animals and tumour lines

LS174T is a human colon adenocarcinoma cell line (ATCC, Rockville, MD, USA) maintained in our lab as a cell culture and serially passed in nude mice. Six- to eight-week-old males of nu/Sed origin were anaesthetized with ketamine (Ketalar, Parke-Davis, Morris Plains, NJ, USA) 100 mg kg–1 body weight, and xylazine (Rompun, Miles Inc, Shawnee Mission, KA, USA) 10 mg kg–1 body weight, and 1-mm3 LS174T tumour chunks were implanted subcutaneously in the right hind leg. Institutional guidelines for animal welfare and experimental conduct were followed.

Experimental set-up

When the tumours became visible, the mice were split into a control group and six experimental groups; controls were fed standard liquid rat diet (LiquiDiet ‘82, Bioserv, Frenchtown, NJ, USA) from special glass liquid diet dispensers. The experimental groups were fed the same liquid diet mixed with different concentrations of Cr (Sigma Chemical, St Louis, MO, USA) and cyCr (Aldrich Chemical, Milwaukee, WI, USA) as follows: 2.5% Cr, 5% Cr, 0.025% cyCr, 0.1% cyCr, 0.5% cyCr and 1% cyCr (all concentrations are w/v). Diet and water were given ad libitum. For 2 weeks the tumours were measured bidimensionally three times weekly. The animals were weighed before and during the feeding, and the volume of diet consumed was read from the diet dispenser. At the end of the 2-week feeding period the animals were anaesthetized with ketamine/xylazine and in vivo 31P-MRS was performed. After spectroscopy, blood samples were taken for determination of serum glucose; tumour and liver samples were taken for determination of total concentrations of CK substrates (Cr + cyCr), lactate and alanine, wet weight/dry weight ratios and CK activity.

Tumour growth curves

Mean tumour growth curves were constructed according to a transformed Gompertz function, as described by Spang-Thomsen et al (1980). For determination of the α-value, a simple transformation of the Gompertz function was used:

\[ \text{Transformed tumour size} = \ln \left[ \ln A(\text{max}) - \ln A(t) \right] = \ln b \alpha - \alpha t \]

where \( \alpha \) and \( \beta \) are constants, \( A(\text{max}) \) is the theoretical maximum size and \( A(t) \) is the tumour size at time \( t \). This growth function depicts a straight line with negative slope (–\( \alpha \)) when the transformed tumour size is plotted against time. In \( A(\text{max}) \) was set to 7, corresponding to tumour dimensions of approximately 15 × 15 mm2. Consequently, all variation in growth is placed in one single parameter, \( \alpha \), that expresses the growth of the individual tumour.

31P-MRS

Spectra were collected with a 300-MHz Bruker Biospec spectrometer (horizontal wide bore) operating at 121.5 MHz for 31P. An 8-mm, two-turn surface coil was located above the tumour. Spectra were collections of 256 transients of 60° pulses at a repetition time of 1 s. Fully relaxed spectra were recorded at a repetition time of 10 s for calculation of the saturation factors for each individual phosphorus resonance (\( n = 10 \)). Free induction decays were multiplied with a Lorentzian window of 30 Hz. Peak areas were integrated with a Bruker software subroutine, after baseline correction. Phosphorus metabolites were quantified against an external reference containing 8 μmol of methylphosphonic acid (MPA) and 20 μmol of gadolinium diethylenetriaminopentacetic acid (gadolinium DTPA). Data are reported as μmol coil–1 because the volume detected by the coil was not quantitated.

Saturation transfer experiments were performed to evaluate magnetization transfer from PCr to nucleoside triphosphate (NTP) (forward CK direction). 31P spectra were collections of 20 transients (90° pulses) with repetition times of 10 s. Magnetization transfer was measured using a narrow-band, low-power radiofrequency (RF) pulse with increasing durations (0.5, 1, 3, 5, 7 and 9 s). The saturating pulse was applied for each duration at the resonance frequency of γ-NTP (\( M_0 \)) and at a symmetrical location downfield from the PCr peak (\( M_p \)). The apparent longitudinal relaxation time (\( T_{\text{unp}} \)) was determined from the mono-exponential decay of \( M / M_0 \) (a pair of saturated/control spectra). Pseudo-first order rate constants (\( k \)) were calculated according to \( k = (1 - M / M_0) / T_{\text{unp}} \). The reaction velocity was calculated from the rate constant and the concentration of PCr (per surface coil detection volume).

H-MRS

1H-MRS was performed on perchloric acid extracts of tumour and liver tissue samples. Spectra were collected with a 300-MHz Bruker spectrometer (vertical narrow bore). Fully relaxed spectra were collections of eight transients of 90° pulses with a relaxation delay of 8 s. After baseline correction, peak areas were integrated with a Bruker software subroutine. Proton resonances were assigned against 1 μmol of trimethylsilylpropionic acid (TSP), which was also used to quantify the metabolites.

Perchloric acid extracts

Tissue samples were diluted with 5 volumes of 5% perchloric acid, homogenized and centrifuged at 15 000 g for 15 min at 4°C. The supernatant was neutralized with potassium hydroxide and centrifuged again at 20 000 g for 20 min at 4°C. The supernatant was lyophilized and stored at –85°C. Extract powder was diluted in 0.6 ml of D2O in the presence of 1 mM ethyleneglycol (bis-aminoethyl ether)tetraacetic acid (EGTA) and 1 μM TSP.

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CK activity

Tissue samples were homogenized in 99 parts of the following buffer: 26 mM Tris, 0.3 M sucrose, 20 mM β-mercaptoethanol and 1% tert.-octylphenoxy-poly(oxyethylene)ethanol (Sigma). CK activity was determined in the direction of Cr to PCr at 37°C by the spectrophotometric assay described previously (Kristensen et al, 1995) and in the opposite direction at 37°C by a commercially available kit (cat. no. 45-1, Sigma).

Gel electrophoresis

CK distribution was tested in samples of tissue homogenate at a final dilution of 1:200. One-microlitre volumes were applied to a 1% agarose Multitrac CK electrophoretic isoenzyme gel (Ciba-Corning, Marshfield, MA, USA) which uses a hexokinase/glucose 6-phosphate dehydrogenase coupled enzyme system.

Serum glucose

Serum glucose was determined spectrophotometrically using a commercially available kit (Sigma Diagnostics, St Louis, MO, USA, cat. no. 510-A).

Tissue water content

Tissue samples were blotted and weighed (ww), and stored for 24 h at 85°C for determination of the dry weight (dw). Total tissue water content was calculated according to (ww–dw) dw⁻¹, and is expressed in units of ml g⁻¹ dw.

RESULTS

Tumour growth

The growth of control tumours and tumours treated with different doses of Cr and cyCr is shown in Figure 1. Both 2.5% and 5% Cr induced significant growth inhibition (P < 0.05) (Figure 1A), whereas the growth inhibition induced by the lowest dose of cyCr, 0.025%, was non-significant. Tumour growth was significantly inhibited by 0.1% and 0.5% cyCr (P < 0.05) (Figure 1B). Figure 2 shows the growth curve of tumours treated with i.p. injections of cyCr (10 mg daily) as compared with controls. Also i.p. injections of cyCr induced significant growth inhibition (P < 0.05).

Toxicity

The animals in all five experimental groups fed Cr or cyCr lost significant amounts of weight during the feeding period compared with controls, and the weight loss was correlated to the mean amount of liquid diet intake (r² = 0.82). There was not, however, any correlation between weight loss and tumour growth inhibition measured as the α-value for each individual tumour (r² = 0.16). The mice fed 0.5% cyCr lost weight rapidly, probably because of the very limited diet intake, and all animals were sacrificed on day 20 because of severe (>30%) weight loss.

Mean serum glucose level after 2 weeks of feeding was 11.5 mM (range 3.2–24.9 mM) in the control group, 11.3 mM (range 6.5–16.2 mM) in the 2.5% Cr group, 14.2 mM (range 11.2–18.2 mM) in the 5% Cr group, 20.1 mM (range 13.2–23.2 mM) in the 0.025% cyCr group and 12.8 mM (range 9.2–15.2 mM) in the 0.1% cyCr group. There was no significant decrease in mean serum glucose concentration in any of the experimental groups compared with controls.

Intraperitoneal injections of cyCr were given in a dose calculated to correspond to the ingested dose of mice fed 0.1% cyCr (10 mg daily i.p.). This dose did not induce significant weight loss or other signs of toxicity when given intraperitoneally.

^3P- and ^1H-MRS

Typical in vivo ^3P- and extract ^1H-MR spectra are shown in Figure 3. Uptake of the substrates by the tumour was evident from the
increase in PCr and PcyCr contents. The absolute levels of NTP and Pi were not significantly different after 2 weeks of feeding with CK substrates, with the exception of the Cr 5% group. At this dietary concentration, Pi concentrations were lower ($P < 0.05$).

None of the experimental groups experienced a decrease in NTP/Pi, and reduction of Pi caused a significant increase in this index only in mice fed with Cr 5% ($P < 0.05$). It can be seen from Table 1 that the PCr/Pi ratio increased in the Cr 5% group ($P < 0.01$) and the PcyCr/Pi ratio increased in both groups fed with cyCr ($P < 0.05$). Acidic intracellular pH values were observed in all of the experimental groups, as compared with controls ($P < 0.002$ and $P < 0.02$ with Cr and cyCr feeding respectively).

Total PCr+Cr and PcyCr+cyCr contents in the tumours increased as a function of the dietary concentration (Table 1). In Cr-fed animals, intratumoral concentrations increased 2.5- and 5-fold at dietary concentrations of 2.5% and 5% Cr. Table 1 shows the levels of PCr and PcyCr detected in different tumours. It was not possible to distinguish PCr from PcyCr in the MR spectrum. The total increase in PCr+Cr was markedly larger than the increase in PCr concentrations. A similar disproportional increase in total vs phosphorylated substrates was observed in tumours of cyCr-fed animals.

There was a strong correlation between Cr+cyCr concentration and degree of growth inhibition ($\alpha$-value) ($r = 0.64$, $P = 0.002$), whereas no correlation was found between growth inhibition and the concentration of phosphorylated compounds ($r = 0.25$, $P = 0.29$) (Figure 4).

Measurements of the magnetization transfer PCr to ATP are presented in Table 2. The reaction velocity was slightly decreased by Cr feeding (not significant). At the higher dose of cyCr, no exchange was detected, indicating that the major fraction of the $^{31}$P resonance was composed of PcyCr. At the lower cyCr dietary concentration, the rate constant of saturation transfer was markedly increased ($P < 0.01$ vs control), indicating the presence of PCr in these tumours. As the relative concentrations of PCr and PcyCr in these tumours were not known, the reaction velocity could not be estimated.

Mean steady-state lactate and alanine concentrations measured by $^1$H-MRS were 24.1 mmol g$^{-1}$ ww and 2.4 mmol g$^{-1}$ ww, respectively,

### Table 1  Metabolite concentrations, pH values and energy indices

|        | $\text{Cr}$ ($\mu$mol g$^{-1}$ ww) | $\text{cyCr}$ ($\mu$mol g$^{-1}$ ww) | $\text{PCr+PcyCr}$ ($\mu$mol col$^{-1}$) | $\text{NTP}$ ($\mu$mol col$^{-1}$) | pH | $\text{Pxx/NTP}$ | $\text{Pxx/P}_i$ | $\text{NTP/P}_i$ |
|--------|-----------------------------------|-------------------------------------|------------------------------------------|----------------------------------|----|-----------------|--------------|--------------|
| Control| 2.0 ± 0.5                          | –                                   | 7.66 ± 1.38                              | 4.96 ± 1.04                      | 7.37 ± 0.07                      | 1.8 ± 0.3    | 2.6 ± 0.7    | 1.4 ± 0.3    |
| Cr 2.5%| 4.8 ± 1.6                          | –                                   | 6.64 ± 1.92                              | 3.44 ± 0.96                      | 6.94 ± 0.19                      | 2.2 ± 0.4    | 2.8 ± 0.7    | 1.3 ± 0.4    |
| Cr 5%  | 10.3 ± 2.4*                         | –                                   | 10.24 ± 1.6*                             | 4.24 ± 0.88                      | 6.99 ± 0.19                      | 2.8 ± 0.1*   | 3.1 ± 1.1*   | 2.2 ± 0.3*   |
| cyCr 0.025%| 3.2 ± 0.9                         | 1.2 ± 0.2*                          | 6.4 ± 1.60                               | 3.6 ± 0.96                       | 7.07 ± 0.10*                     | 2.2 ± 0.2    | 3.5 ± 0.3*   | 1.6 ± 0.1    |
| cyCr 0.1%| 1.8 ± 0.4                          | 6.2 ± 0.1*                          | 9.04 ± 2.32                              | 3.92 ± 0.96                      | 7.00 ± 0.05*                     | 2.6 ± 0.4*   | 4.3 ± 0.3*   | 1.7 ± 0.3    |

Values are given as means ± s.d. Pxx represents PCr and/or PcyCr. *$P < 0.05$ vs control.
Table 2  Pseudo-first-order rate constants (k) and reaction velocity (v) of phosphate transfer from PCr to NTP in the four experimental groups

|        | k (s⁻¹) | v (mmol coil⁻¹ min⁻¹) |
|--------|---------|-----------------------|
| Control| 0.18 ± 0.03 | 1.04 ± 0.18          |
| Cr 2.5%| 0.15 ± 0.02 | 0.75 ± 0.2           |
| Cr 5% | 0.12 ± 0.02 | 0.92 ± 0.14          |
| cyCr 0.025% | 0.31 ± 0.04* | –                   |
| cyCr 0.1% | 0*       | 0*                   |

*P < 0.05 vs control.

**Energetic considerations**

After 2 weeks of oral feeding with Cr or cyCr, the tumours showed no signs of energy deprivation measured as PCr+PcyCr/NTP and NTP/P, Cr and cyCr feeding increased the concentrations of the phosphorylated Cr substrate, without significant changes in NTP and P, levels. It is evident that the increase in PCr+PcyCr/NTP was caused by enrichment of the tumours with Cr and cyCr (Table 1). The phosphorylation potential ([ATP][ADP]–1 [Pi]–1) is important for regulation of oxidative metabolism and can be altered by changes in the relative concentrations of substrates and products in the CK equilibrium. At equilibrium, changes in the concentrations of Cr or cyCr and PCr or PcyCr will influence the concentrations of ATP, ADP and H⁺ according to the equilibrium constant (Keq):

Keq(Cr) = [ATP][Cr][ADP][PCr][Pi][H⁺]

Keq(cyCr) = [ATP][cyCr][ADP][PcyCr][Pi][H⁺]

In our study, the increase in Cr exceeds the increase in PCr, possibly leading to a decrease in ADP concentration, which will increase the phosphorylation potential and decrease the rate of oxidative metabolism. In contrast, the acidification of tumours may lower the ADP concentration (provided that all other concentrations are unchanged) and increase the oxidative phosphorylation rate. Thus, changes in oxidative phosphorylation/glycolysis rate may not be reflected in the MRS-detectable energetic ratios PCr+PcyCr/NTP and NTP/P. Unfortunately, calculation of the phosphorylation potential was not possible in these experiments.

The apparent increase in PCr+PcyCr/NTP ratio does not necessarily translate into an improved energetic state, and the tumours may actually be energy deprived as PcyCr is 30-fold more stable than PCr, and its turnover is 160 times less efficient in vitro (LoPresti et al, 1989). Consequently, the NTP/P ratio may be better than the PCr+PcyCr/NTP ratio as an indicator of the energetic state of the tumour in the absence of ADP concentrations and calculations of phosphorylation potential. NTP/P was not significantly affected in any of the experimental groups except for the group fed 5% Cr, in which its increase was the result of lower Pi concentrations (Table 1). Growth inhibition does not seem to be caused by energy deficiency measured as NRS or PcyCr, but other energetic effects not reflected in these ratios cannot be excluded.

It is evident from the present results that Cr and cyCr had a direct cytostatic effect on tumour cells, as previously demonstrated in vitro (Schiffenbauer et al, 1996). Growth inhibition was associated with tumour acidification, starting from relatively alkaline pH values in controls, characteristic of most untreated tumours (Griffith, 1991). The effect of Cr and cyCr may be mediated by lactate accumulation secondary to inhibition of oxidative phosphorylation. However, the low pH values were not accompanied by high intratumoral lactate concentrations. The observation that lactate concentrations were not raised further supports the concept...
that energy metabolism was not impaired. In parallel, high plasma glucose levels indicate ample substrate delivery to the tumour, and the stable NTTP/Pi ratio suggests that the tumours were adequately perfused. This index was shown to be a sensitive detector of changes in blood flow in ex vivo mammary adenocarcinoma preparations; in this tumour NTTP/Pi was most sensitive to changes in carbon substrate supply and less to oxygen delivery (Eskey et al., 1993). The present experiments address directly only chronic effects of Cr and cyCr administration, via an oral route, in tumour-bearing mice. Our findings corroborate the results of Schiffenbauer et al. (1996), who found no changes in NTP levels and a gradual increase in PcyCr during 12–20 h of acute cyCr administration in vitro.

The undetectable phosphate transfer rate between PCr and γ-NTP in the 0.1% cyCr group indicated that this tumour contained almost exclusively PcyCr. The turnover of phosphate between PcyCr and NTP is slower than the nuclear magnetic resonance (NMR)-detectable time scale for saturation transfer. It follows, from the increased rate of saturation transfer in the 0.025% cyCr group, that, whenever present, PCr was the donor/acceptor of high-energy phosphate. Although the kinetic properties of PcyCr may induce changes in intracellular environment, such as lowering ADP levels, its chronic administration did not decrease NTTP/Pi or total NTP concentration (Table 1). Chronic cyCr administration allowed the replenishment of P_i, as there were no signs of a decrease in P_i concentration in any of the cyCr-treated groups.

Growth inhibition by Cr and cyCr

In the present experiments, we found a close relationship between growth inhibition and concentration of Cr and cyCr (Figure 4 and Table 1) that was consistent with a direct toxic effect of these CK substrates. In contrast to the total substrate concentrations, there was a very poor correlation between growth inhibition and the steady-state intratumoral concentration of PCr and PcyCr (Figure 4). Thus, the concentration of substrate seems to be more important than the concentration of phosphorylated products for the growth-inhibitory effect of both compounds. Intracellular ‘trapping’ of Cr and cyCr due to phosphorylation may well occur and would explain the increased effect of cyCr in tumours with high CK activity and after transfection of cancer cells with the CK gene (Lillie et al., 1993). This possibility is further supported by the fact that there seems to be a steep dose–response curve in the dose range applied in the present experiments (Figure 1) and in experiments by other investigators (Lillie et al., 1993; Miller et al., 1993).

It is noteworthy that the total tumour concentrations of cyCr and Cr were comparable (6.2 ± 0.1 in the 0.1% cyCr group compared with 10.3 ± 2.4 in the 5% Cr group), despite a dietary Cr/cyCr ratio of 50:1 (Table 1). These numbers indicate a preferential uptake of cyCr into the tumour tissue. The role of a specific sodium-dependent transporter of Cr and cyCr (Guimbal et al., 1993; Sora et al., 1994) remains to be determined. This transport molecule may have a higher affinity for cyCr than Cr. Estimates indicate that 48–67% of Cr transport from interstitial fluid to the intracellular space is sodium dependent at [Na+] = 155 mM and [cyCr] = 5 mM (Schiffenbauer et al., 1996). At cyCr concentrations much greater than the estimated $K_m$ value of 0.54 mM, passive diffusion becomes more important. As cyCr is found, in this and other (Schiffenbauer et al., 1996) studies, to be active at a concentration of 5 mM, the cotransport of sodium and Cr/cyCr may be involved in the mechanism of action of these compounds. The in vivo growth-inhibitory effect was achieved at an oral dose of cyCr 5–10 times lower than in the studies by Lillie et al. (1993) and Miller et al. (1993), whereas the effective i.p. dose in this study (10 mg daily) was comparable to the i.p. dose used by others (Teicher et al., 1995; Schimmel et al., 1996). However, the intratumoral substrate concentration of 5.0 mM reached in the 0.1% cyCr group (Table 1) was very close to the reported in vitro growth-inhibitory levels of 7–20 mM (Martin et al., 1994a). The fact that growth inhibition was observed at similar concentrations in vitro and in vivo demonstrates that the effect of cyCr on these tumours is not caused by collapse of stromal components, e.g. tumour vasculature.

Involvement of CK in growth

Schiffenbauer et al. (1996) examined two different cell lines for cyCr content; the cyCr-sensitive rat glioma cell line C6 had a CK activity of 0.16 unit mg⁻¹ and a dramatic increase in intracellular PcyCr content during cyCr perfusion, whereas the CK activity of the less sensitive ovarian carcinoma line OC238 was tenfold lower and did not seem to accumulate either PcyCr or cyCr. These observations may be explained by slower phosphorylation in the OC238 cells (as a result of the lower CK activity), leading to a slower accumulation of the total amount of cyCr in these cells.

The CK activity of 2.1 units mg⁻¹ is one order of magnitude higher than that of the tumour line with the highest activity in the study by Schiffenbauer et al. (1996), and exactly the same as the activity previously reported for the colon adenocarcinoma cell line Caco-2 (Lillie et al., 1993). The relatively high CK activity in LS174T may explain the very high sensitivity of these tumours to Cr and cyCr at very low doses. On the other hand, the CK activity will only influence the phosphorylation rate and not the intracellular concentration of PCr and PcyCr, provided that the system is in equilibrium. Thus, once the steady-state concentration of PCr/PcyCr is reached, the CK activity of the tumour cells may be less important than the given dose or the plasma concentration of substrate. It is unknown whether the Cr transporter is expressed in parallel or is independent of CK.

A recent study has shown that subcellular localization of the isoenzymes may be of importance for hepatocyte regeneration, as liver regeneration was impaired in transgenic mice with livers expressing CK-mit fed Cr and in mice with livers expressing CK-B fed cyCr (Askensy and Koretsky, 1997). CK-B is the most abundant isoenzyme in most tumours (DeLuca et al., 1981; Lillie et al., 1993) and fetal tissues (Hall and DeLuca, 1976). In LS174T, only cytosolic isoenzymes of CK were detected with an expression of ≈90% BB and ≈10% MB. The association of CK-B with growth, and the increased sensitivity of growing tissues containing this isozyme to cyCr, suggests that it may be worthwhile to investigate the isoenzyme distribution of tumours for two purposes: (1) to optimize the anti-tumour effect of Cr and cyCr and (2) to elucidate the interaction between enzymes and substrates in the mechanism of action of Cr and cyCr. The isozyme distribution in LS174T tumours (≈90% BB) is very close to the distribution in colon washings from normal subjects (Berezniatsky et al., 1982).

Water accumulation

Neither Cr nor cyCr induced significant water accumulation in the tumour tissue in spite of significant growth inhibition (Figure 5). In contrast, both treatment modalities increased the total liver water content significantly, thus corroborating previous studies of
water accumulation in liver tissue after Cr and cyCr treatment (Askenasy and Koretsky, 1997). However, in that study, the induced hepatic oedema could not explain the inhibitory effect on liver growth. Correspondingly, we did not find evidence that the tumour growth-inhibitory effect is attributable to intratumoral water accumulation in vivo, although this mechanism has been suggested to be of importance for inhibition of in vitro tumour cell proliferation (Schiffenbauer et al, 1995). Similarly, the induction of oedema in itself could not explain the growth-inhibitory effect of cyCr on C6 or OC238 cells (Schiffenbauer et al, 1996).

Systemic effects

Severe fasting and weight loss has previously been shown to inhibit tumour growth (Giovannella et al, 1982), but in the present experiments the lack of correlation between weight loss and growth inhibition indicated that weight loss was not the direct cause of growth inhibition. This is further corroborated by the observation that i.p. injections of cyCr did not induce weight loss, but did inhibit tumour growth (Figure 2), at a dose (10 mg i.p. daily) directly comparable to the amount of cyCr ingested by the mice fed 0.1% cyCr. Finally, because both colon tumours (Bartholomew and Schutt, 1971) and cyCr feeding cause mild hypoglycaemia (unpublished results), plasma glucose levels were measured. In the absence of significant differences in the experimental groups, growth inhibition cannot be attributed to the non-specific effects of starvation.

In this study, Cr and cyCr for feeding experiments were mixed in a viscous liquid diet instead of dry food. In previous experiments performed in our lab, we observed that the mice were able to avoid the cyCr crystals when mixed with powdered dry food. Consequently, the effective dose may be smaller than was assumed in the previous experiments by Lillie et al (1993) and Miller et al (1993). Also, the mouse strain may be of importance – nude mice seem to tolerate less cyCr than immunosufficient mice and there might be a difference in tolerance between different strains of nude mice. It is possible that the effect of Cr and/or cyCr is mediated by acidification of the tumour tissue, but the exact mechanism of action of these compounds remains unknown. Further studies are warranted to elucidate the role of CK isozymes on the anti-tumour effect and to optimize treatment schedules for these drugs alone or combined with synergistically acting chemotherapeutic agents (Teicher et al, 1995).

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