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Microtubule stabilization and potentiation of taxol activity by the creatine analog cyclocreatine

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Creatine kinase (CK), a key enzyme of cellular energetics, has been implicated in tumorigenesis. Cyclocreatine (CCr), which forms a stable phosphagen with a reduced rate of ATP regeneration through CK, inhibits the growth of many solid tumors. We report that CCr induces the formation of unusually stable microtubules that resist depolymerization by nocodazole. By reducing ATP availability, CCr may modulate the activity of kinases that regulate microtubule dynamics. Further, combinations of CCr and taxol resulted in the synergistic killing of breast tumor cells indicating that CCr may be a useful addition to chemotherapy's that include taxanes.

Key words: Combination chemotherapy, creatine kinase, cyclocreatine, microtubules, taxol.

Introduction

Creatine kinase (CK) and its substrates, creatine and creatine phosphate (Cr-P), comprise a system that insures the rapid regeneration of ATP in tissues with high and fluctuating energy demands, such as cardiac and skeletal muscle, brain, photoreceptors and spermatozoa (for a review, see Wallimann *et al.*[1]). The enzyme reversibly catalyzes the transfer of the phosphoryl group G-P to ADP, thus producing creatine and ATP. Isoenzymes of CK are localized at distinct subcellular sites. The mitochondrial forms of CK (CK-Mi) are located at sites of energy production, while the cytosolic isoenzymes, the skeletal muscle (CK-MM), heart (CK-MB) and brain (CK-BB) forms, tend to be located at sites of cellular work. The creatine kinase/creatine phosphate (CK/Cr-P) system is thought to play a central role in the transport and buffering of cellular energy.[1] Other roles include preventing accumulations of ADP and protons in cells and maintaining appropriate local ATP ADP ratios. The CK/Cr-P system is also functionally coupled through P_i production and Cr-P levels with cellular metabolic pathways, including glycogenolysis and glycolysis. It has been proposed to closely couple ATP demand and synthesis in some tissues as a result of the association of CK-Mi with the ATP–ADP translocase.[2,3]

CK, especially the brain isoform, appears to play an important role in the process of tumorigenesis.[4,5] CK-BB has been found at elevated levels in a broad spectrum of tumors, including small cell lung, colon and prostate carcinomas.[5,6] Its over-expression has been associated with progressive metastatic disease and with a poor patient prognosis. CK-BB activity or expression is increased by several hormones, cell signal transducers and an oncogene.[4,5] It has been proposed that the presence of CK isozymes in tumor cells could be a necessity for energy distribution, rather than an adventitious genetic deregulation due to the cancer process.[7]

Cyclocreatine (CCr), a substrate analog of CK first synthesized and studied in the early 1970s,[8] has more recently been shown to inhibit the growth of a broad spectrum of solid tumors both *in vitro* and *in vivo*.[5,9–12] CCr is readily phosphorylated by CK to create the new synthetic phosphagen, CCr-P.[9,13–15] Though structurally similar to its analog Cr-P, CCr-P has distinct thermodynamic and kinetic properties that result in its intracellular accumulation to higher concentrations than the natural phosphagen.[16–18] Using V_{max}/K_M as a measure of substrate quality, the new phosphagen is turned over 16-fold less efficiently than Cr-P and hence generates ATP at a reduced rate.[14,15] Exposure of CK-expressing tumor cells to CCr results in the build up of the phosphorylated form of the compound[9,12,17] and it appears that only tumor cells high in CK are sensitive to the compound.[9] Hence it has been proposed that CCr acts as an anticancer agent by reducing energy availability to ATP-requiring processes critical for tumor cell proliferation.[9] Alternatively, CCr-P may represent an activated form of the compound that accumulates to high levels and modulates unidentified cellular processes.

The cytosolic CK isoenzymes have been observed to associate with the cellular cytoskeleton. For ex-

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ample, CK-MM and CK-MB are tightly associated with the M-band of actin myofibrils,[19] where they function to maintain energy homeostasis during muscular activity (for a review, see Wallimann *et al.*[1]). Indirect evidence suggesting an association between CK and cellular microtubules and intermediate filaments has also been presented, and includes the results of immunolocalization, *in vitro* binding and functional studies performed to investigate the cytosolic CK isoenzymes.[20–24] Based on these observations, we have examined the effects of CCr on the organization of microtubules in interphase cells. Results are presented for two CCr-sensitive human tumor cell lines, the cervical carcinoma ME-180 and the breast adenocarcinoma MCF-7.

Materials and methods

Drugs, cell lines and cell culture

CCr was synthetized as described.[25] It was dissolved at 14, 28 or 56 mM in complete media by incubating at 37°C for 15 min then rocking 1–2 h at room temperature. Solutions were stored at 4°C and used within 1 week. Taxol was a gift from Bristol–Meyers–Squibb (Wallingford, CT). ME-180 cells were derived from an omental metastasis of a rapidly spreading cervical carcinoma.[26] They were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in EMEM complete medium: minimum essential medium with Earles balanced salts without glutamine, supplemented with 1 mM sodium pyruvate, 4 mM glutamine, 10% fetal bovine serum and 0.1 U/ml penicillin/streptomycin (all JRH Biosciences). MCF-7 breast adenocarcinoma cells were derived from breast pleural effusion[27] and obtained from Southern Research Institute (Birmingham, AL). They were maintained in EMEM complete medium with 10 μg/ml bovine insulin (Sigma, St Louis, MO). MRC-5 fibroblast cells were derived from normal fetal human lung tissue.[28] They were obtained from the American Type Culture Collection and maintained in EMEM complete medium.

Nocodazole challenge experiments

Cells growing exponentially on glass coverslips in 6-well plates were incubated under normal tissue culture conditions in 3 ml of complete media per well with or without CCr for the indicated times. A 1 mg/ml stock solution of nocodazole (Sigma) in dimethylsulfoxide was serially diluted into complete media with or without CCr. Media on cells was replaced with 3 ml nocodazole-containing media and cells were incubated under normal tissue culture conditions for 1 h, during which time they were exposed to the same CCr concentration as previously. Following nocodazole treatment, cells were immediately stained for α-tubulin by indirect immunofluorescence and photographed.

Indirect immunofluorescence

Following drug treatment, cells on coverslips were rinsed twice with Dulbecco's phosphate buffered saline (pH 7–7.2, DPBS) and fixed with glutaraldehyde for 10 min at room temperature in 0.3% glutaraldehyde, 0.5% NP-40, 5 mM EGTA, 1 mM $MgCl_2$ in 80 mM PIPES, pH 6.8. Coverslips were then rinsed twice in DPBS, neutralized with sodium borohydride at 10 mg/ml in DPBS for 7 min, followed by 0.1 M glycine in DPBS for 20 min. After another DPBS rinse, coverslips were washed four times in reaction buffer: 1% BSA, 0.1% Tween-20 in DPBS, for a total of 20 min. The primary antibody (DM1A for α-tubulin, clone 2.1 for β-tubulin, both from Sigma) was added in reaction buffer and allowed to incubate for 30 min at room temperature. After washing three times in reaction buffer for a total of 15 min, the secondary antibody, goat anti-mouse $F(ab')_2$ FITC-conjugated antibody (Cappel), was added and coverslips were incubated for 30 min at room temperature. Coverslips were rinsed again in reaction buffer, then mounted in *p*-phenylenediamine and glycerol (pH 8.6).[29]

Slides were viewed and photographed on a Nikon Dialphot-TMD inverted microscope equipped for epifluorescence using an excitation wavelength of 470–490 nm, a Nikon N8008S camera and Kodak ASA 100 color print film. A ×60 PlanApo objective was used with ×1.5 camera magnification to give ×150 total magnification for all photographs presented.

Microtubule repolymerization experiments

ME-180 cells were pretreated under normal tissue culture (TC) conditions with or without 14 mM CCr for 24 h. Cells were then transferred to medium containing 1.5 μg/ml nocodazole with or without CCr at the same concentration as previously and incubated under normal TC conditions for 1 h to

depolymerize microtubules. Cells were then transferred to nocodazole-free medium that contained CCr at the same concentration as previously and microtubules were allowed to repolymerize for 5 min under normal TC conditions. Cells were then stained for α-tubulin by indirect immunofluorescence and photographed.

Combination colony assays

For combination studies against the breast adenocarcinoma cells, a concentration of 500 μM CCr was used. Combination regimens consisted of a 24 h exposure of the cells to CCr in media without serum with a 1 h exposure to a range of concentrations of taxol during the fifth hour. Thus CCr was present prior to, during and after treatment with taxol. Following drug exposure, cells were washed three times with phosphate buffered 0.9% saline solution and then plated in duplicate at three dilutions in monolayer for colony formation. Results are expressed as the surviving fraction of treated cells as compared with the vehicle-treated control cells. Values represent the mean ± SEM of three repeated experiments.

For additivity analysis, isobolograms (envelopes) were generated for the special case in which the dose of one agent is held constant.[30] Combinations producing an effect within the envelope boundaries were considered to be additive and those displaced to the left were considered greater-than-additive (synergistic).

Results

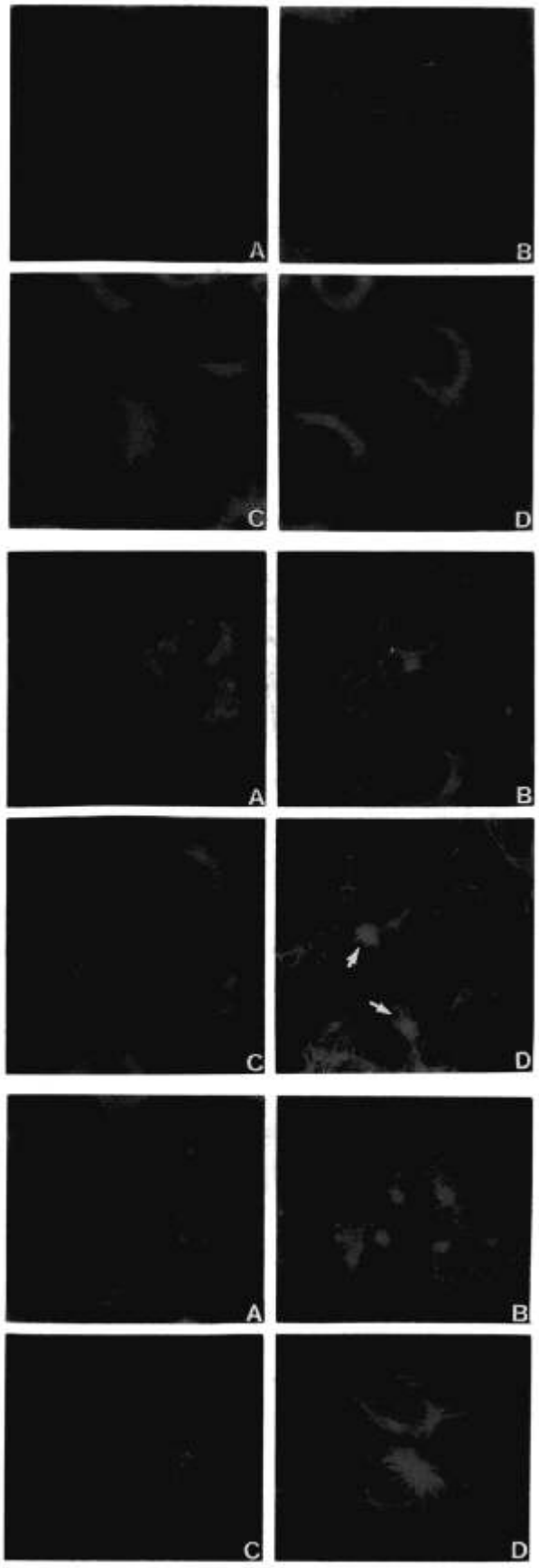
Previous studies have shown that CCr inhibits the growth of a variety of established tumor cell lines with 50% inhibitory concentrations (IC_{50} values) in the low millimolar range (0.8–9 mM).[9,12] Using a soft agar colony assay in which cells were continuously exposed to CCr during the 21 day period of colony formation, the IC_{50} value determined for human ME-180 cervical carcinoma cells was 2.2 ± 0.4 mM[9,12] and for human MCF-7 breast adenocarcinoma cells was 1.7 ± 0.2 mM. CCr has been shown to block tumor cell proliferation without disrupting cellular integrity.[12] The proliferation block is initially reversible (after 8–24 h of exposure), but becomes irreversible upon longer periods of CCr exposure.[12]

To study the effect of CCr on microtubules, tumor cells were treated for 38 or 48 h with the minimum concentration of CCr that completely prevents their proliferation: 14 mM for ME-180 cervical carcinoma cells[12] and 28 mM for MCF-7 breast adenocarcinoma cells. Microtubules were then visualized by indirect immunofluorescence. Results show that CCr treatment caused the microtubules to become more randomly organized (Figure 1), an effect most apparent at the periphery of the cervical carcinoma cells. A morphological change accompanied the microtubule changes; cells appeared to flatten and loose their bipolar shape. Comparable results were obtained with two other CCr-sensitive human tumor cell lines studied, the prostate adenocarcinoma DU145 and the cervical carcinoma SiHa (data not shown).

The results of control experiments indicated that the stained structures represent microtubules. Indirect immunofluorescence of untreated and CCr-treated ME-180 cells using the secondary antibody alone or a CD4-specific primary antibody as a negative control showed no staining (data not shown). Also, results obtained using an anti-β-tubulin antibody (data not shown) were identical to those using the anti-α-tubulin antibody. All experiments reported were repeated at least twice with comparable results.

To address the mechanism responsible for the altered organization of microtubules, we performed a challenge experiment using nocodazole. This agent rapidly crosses the cell membrane and induces microtubule depolymerization in a manner apparently similar to colchicine, which binds to tubulin molecules and prevents their polymerization.[31] Nocodazole causes the rapid depolymerization of normal microtubules.[32,33] Cells were treated with CCr then exposed to 0.25–3.0 μg/ml nocodazole as specified for 1 h. Microtubules remaining were visualized by staining with anti-α-tubulin and were compared to microtubules remaining in cells not treated with CCr.

Treatment of the cervical carcinoma cells with CCr for 13, 24 or 38 h induced the formation of many nocodazole-resistant microtubules (Figure 2). The average length of these resistant microtubules appeared to increase as a function of time of cell exposure to CCr. Under identical conditions, ME-180 cells not treated with CCr contained essentially no microtubules. Thus, CCr induced the formation of microtubules that were more stable when challenged with nocodazole than were normal microtubules. Further, several characteristics of the CCr-stabilized microtubules were unusual. These microtubules were short, randomly organized and apparently unassociated with the centrosome. In



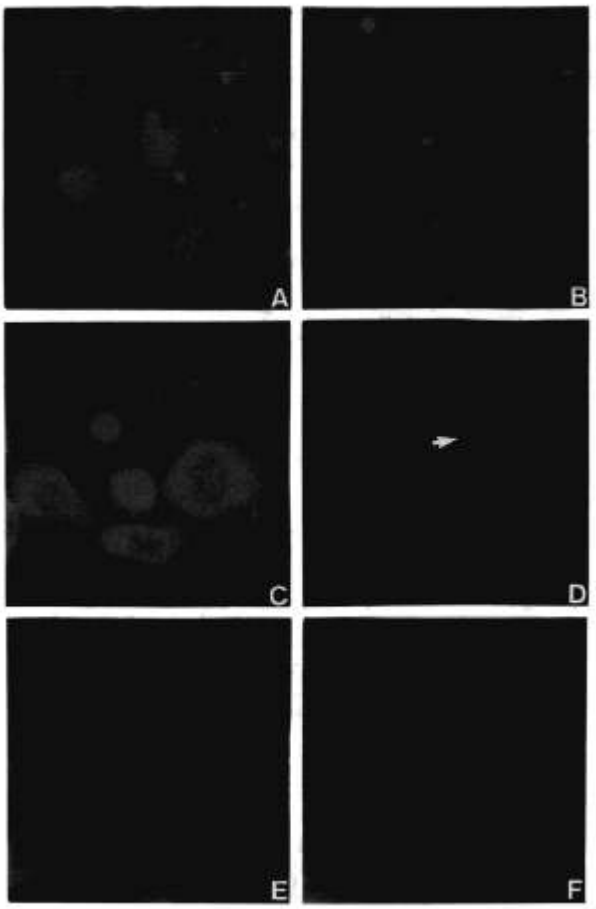


Figure 1. Effect of CCr on the organization of microtubules in two CCr-responsive tumor lines. ME-180 cervical carcinoma cells: (A) untreated, (B) 14 mM CCr for 38 h. MCF-7 breast adenocarcinoma cells: (C) untreated, (D) 28 mM CCr for 48 h. Cells were treated with CCr then stained for α-tubulin by indirect immunofluorescence and photographed.

Figure 2. Effect of CCr on microtubule stability of ME-180 cervical carcinoma cells determined by nocodazole challenge. Cells were treated with or without CCr followed by nocodazole: (A) 0.5 μg/ml nocodazole, (B) 14 mM CCr for 13 h then 0.5 μg/ml nocodazole, (C) and (E) 1.5 μg/ml nocodazole, (D) 14 mM CCr for 24 h then 1.5 μg/ml nocodazole, (F) 14 mM CCr for 38 h then 1.5 μg/ml nocodazole. Arrows indicate centrosomes.

Figure 3. Effect of CCr on microtubule stability of MCF-7 breast adenocarcinoma cells determined by nocodazole challenge. Cells were treated with or without CCr followed by 1.5 μg/ml nocodazole: (A and C) nocodazole alone, (B) 28 mM CCr for 24 h, (D) 28 mM CCr for 48 h. Arrows indicate centrosomes.

Figure 4. Effect of CCr on microtubule repolymerization. Tumor cells were pretreated with or without CCr for 24 h. Microtubules were disrupted with nocodazole, then allowed to repolymerize briefly with or without CCr. ME-180 cervical carcinoma cells: (A) no CCr, (B) 14 mM CCr. MCF-7 breast adenocarcinoma cells: (C) no CCr, (D) 28 mM CCr.

contrast, it is generally believed that all normal cellular microtubules originate only at the centrosome from which point they radiate out to the cell periphery.[34,35] Thus, CCr apparently induced the formation of an aberrant population of new microtubules that were unusually stable.

Control experiments as described above, but performed after treatment of ME-180 cells with nocodazole and CCr, indicated that the nocodazole-resistant structures represent microtubules (data not shown). In addition, there was no change in the amount of α-or β-tubulin in ME-180 cells after treatment for 48 h with CCr as assayed by immunoblotting (data not shown).

The timing of microtubule stabilization by CCr was similar to that of the earliest CCr-induced effects previously described. In previous studies, inhibition of cell cycle progression was detected after 8–16 h of CCr exposure.[23] CCr (and CCr-P) required the same length of time to accumulate to half-maximum concentrations in tumor cells.[12]

To determine whether CCr induces the formation of nocodazole-resistant microtubules in other tumor cell lines, we examined the CCr-sensitive human breast adenocarcinoma MCF-7. Cells were treated with CCr then challenged with nocodazole. As in the cervical tumor line, nocodazole-resistant microtubules were observed that were disorganized and apparently not attached to the centrosome (Figure 3). An additional population of nocodazole-resistant microtubules was apparent in the CCr-treated breast tumor cells that was not as apparent in the cervical cells. Microtubules extended from each centrosome, making them appear as bright aster-like structures (Figure 3, see arrows). These may be remnants of the microtubule network that existed prior to CCr treatment. Thus, CCr treatment may have rendered the existing network more stable. Alternatively, it may have caused stable new microtubules to form at the centrosome.

To further examine the effect of CCr on microtubules, microtubule repolymerization experiments were performed. Microtubules of ME-180 cervical tumor cells were dissociated by exposing the cells to nocodazole. Nocodazole was then removed from the cells and microtubules were allowed to repolymerize briefly in the presence or absence of CCr. In this protocol, cells were pretreated for 24 h with CCr to allow time for uptake and phosphorylation of the compound. CCr was present during nocodazole treatment and the subsequent repolymerization period. The presence of CCr resulted in the repolymerization of a more extensive array of microtubules than that which formed in the absence of CCr (Figure 4). The newly polymerized microtubules appeared to originate from the centrosome.

To test whether microtubule stabilization is specific to CCr-sensitive tumor cells, we examined the effect of the agent on a CCr-resistant, non-transformed cell line using the nocodazole-challenge experiment. Non-transformed cell lines, which generally express low levels of CK, have been shown to be resistant to CCr.[9,36] For example, proliferation of the normal human fibroblast cell line MRC-5[28] is not affected by CCr.[36] When these cells were examined in the nocodazole challenge experiment, no effect of CCr on interphase microtubules was observed under conditions used for the tumor cells (data not shown). Further, no microtubules were observed when the CCr concentration was increased to 56 mM and when the nocodazole concentration was titrated down to the point where some microtubules remained in untreated controls (data not shown).

Since both CCr and taxol increase the stability of cellular microtubules, we examines the activity of the two agents used in combination. For combination studies, human MCF-7 breast adenocarcinoma cells were treated *in vitro* with 500 μM CCr for 24 h, a dose that was only slightly cytotoxic. The CCr/taxol regimen consisted of a 24 h exposure of the cells to CCr with a 1 h exposure to taxol at the fifth hour so that CCr was present prior to, during and after taxol treatment. Survival curves for exponentially growing MCF-7 cells after 1 h exposure to taxol are shown (Figure 5). The combination of CCr and taxol resulted in greater-than-additive (synergistic) killing of the cells with the greatest synergy at high concentrations of taxol.

Discussion

The results of studies reported here show that the new anticancer agent CCr induces the formation of unusually stable microtubules. These microtubules resist depolymerization induced by the microtubule poison nocodazole. Stable microtubules were induced by CCr in the two tumor cell lines studied, human ME-180 cervical carcinoma and MCF-7 breast adenocarcinoma cells.

Microtubule stabilization by CCr correlated with the anticancer activity of the compound since stabilization was observed in two CCr-sensitive but not a CCr-insensitive cell line. In addition, CCr induced microtubule stabilization after an exposure period comparable to the minimum time required for the inhibition of cell cycle progression (13 h).[12] This

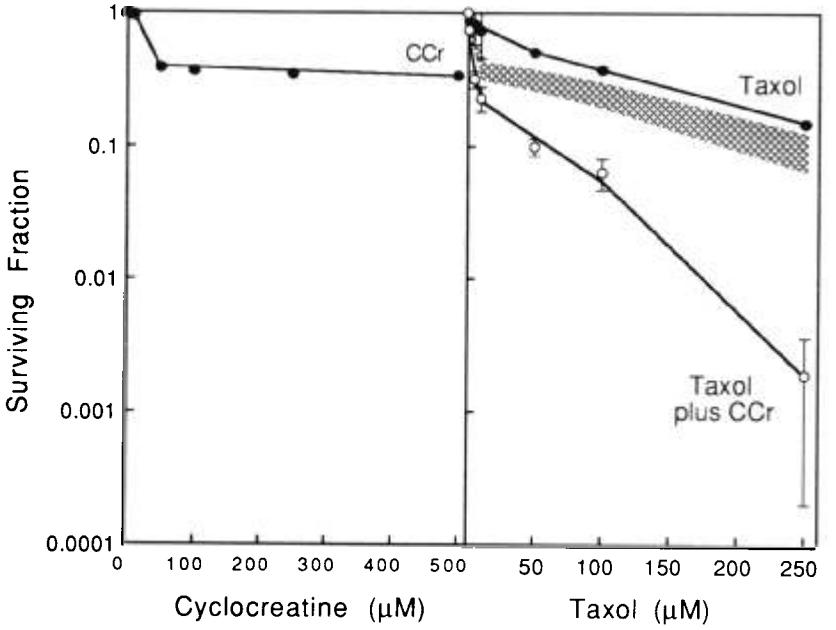


Figure 5. Synergistic cytotoxicity of CCr and taxol against human MCF-7 breast adenocarcinoma cells. (A) Survival of exponentially growing MCF-7 cells exposed to 50–500 μM CCr for 24 h.[27] (B) Survival of MCF-7 cells exposed to 500 μM CCr for 24 h with exposure to various concentrations of taxol for 1 h during the fifth hour of CCr treatment (○). Survival of cells exposed to taxol alone (●). Cross-hatched area, envelope of additivity; shaded area, clinically achieved serum concentration of taxol; points, means of three experiments; bars, SE.

period of time corresponds to that required for the accumulation of CCr to half-maximum levels in cells.[12] This correlation suggests that the antiproliferative activity of CCr may result from its effects on microtubules.

CCr induced the formation of an aberrant population of microtubules that apparently did not originate at the centrosome. This suggests that the compound reduces the critical concentration of tubulin (the threshold concentration required for microtubule assembly). It is generally held that drugs that decrease the critical concentration of tubulin abrogate the effect of the centrosome and induce the assembly of free microtubules in cells.[35,37] Noncentrosomal microtubules have been shown to form in cells under several conditions that lower the critical concentration of tubulin, including the presence of taxol, reduced pH and agents that deplete cellular ATP.[37,38]

CCr represents a second anticancer molecular structure, in addition to the taxanes (38–40), that increases the stability of cellular microtubules. Taxol acts as an anticancer agent by binding directly to tubulin, lowering its critical concentration, and increasing the stability of microtubules *in vitro* and in cells (for a review, see Manfredi and Horwitz[41]). Taxol-treated microtubules, like those treated with CCr, are particularly resistant to conditions that increase the critical concentration of tubulin and usually promote depolymerization.[38,39] Also like CCr, taxol induces the formation of microtubules that do not originate at the centrosome.[38]

Though CCr is similar to taxol in inducing the formation of stable microtubules, perhaps by reducing the critical concentration of tubulin, its effects differ from taxol in several aspects. First, while taxol stabilizes existing interphase microtubules,[38] we have detected only very minor effects of CCr on existing microtubules. The predominant effect of CCr was to induce the formation of what appeared to be newly formed and highly stable microtubules. The stabilization of existing microtubules by taxol provided an early indication of the drug's binding site on the polymer.[38,42] Thus, the lack of an effect on existing microtubules by CCr may indicate the absence of direct binding by the compound. Second, taxol induces extensive arrays of microtubules aligned in parallel bundles,[38,40] an effect not observed with CCr. Thus, while CCr and taxol may both act as anticancer agents by reducing the critical concentration of tubulin and stabilizing cellular microtubules, their mechanisms of action and the specific characteristics of their effects on tumor cells are likely to differ.

The effects of CCr on cell cycle progression have recently been described.[12] Alterations include a

general inhibition of progression out of all phases of the cycle and an accumulation of some cells in G_2/M phase. By analogy with the G_2/M phase accumulation induced by other agents that interact with microtubules, the CCr-induced G_2/M phase accumulation may result from microtubule stabilization. The general inhibition of cell cycle progression out of all phases could also be due to the microtubule effects of the compound, though this is without precedent. Microtubules are known to be critical for many vital interphase functions, including the maintenance of cell shape, cellular motility and attachment, intracellular transport and cell signaling pathways.

To explain the mechanism of stable microtubule formation by CCr, we propose that, by reducing the rate of ATP regeneration through CK, the compound may modulate the activity of proteins that regulate microtubule dynamics in tumor cells. Experiments with ATP-depleting agents have shown that energy is critical for the maintenance of appropriate microtubule dynamics.[37,42,43] The general depletion of total cellular ATP by the metabolic inhibitors azide and 2-deoxyglucose has been reported to protect microtubules against depolymerization. It has been proposed that ATP depletion inhibits the phosphorylation of microtubule-associated proteins (MAPs), increasing their affinity for, and thus stabilizing, microtubules.[37,42,43] In addition, ATPases such as katanin[44] apparently participate in microtubule disassembly. The rates of ATP production need to be addressed in the context of CCr-responsive tumor cells.

Unlike CCr, the metabolic inhibitors that deplete ATP and increase microtubule stability act as nonspecific cytotoxins. CCr has the distinction of apparently modulating energy availability and interfering with microtubule dynamics specifically in tumor cells. Tumor specificity of CCr may in part be explained by CK expression patterns. Studies performed to evaluate the effect of CCr against non-transformed cell lines have found that many established normal lines express low levels of CK and, as would be predicted, are not sensitive to CCr.[9,45] CK levels in only a relatively small number of human tissues are comparable to the elevated levels found in tumors: high CK tissues include skeletal and heart muscle, while significantly lower levels of the enzyme are found in brain, urinary bladder, stomach and colon.[46] The general observation that animals tolerate the administration of high doses of CCr:[9–11,15,16] however, suggests that the compound has little or no detrimental effect against any normal tissues, including those that are high in CK activity. Thus it appears that CCr may be able to specifically target transformed cells.

In conclusion, the evidence presented shows that CCr induces the formation of unusually stable microtubules. Similar to taxol, an anticancer drug that also stabilizes microtubules, CCr may be cytotoxic to tumor cells through its effects on microtubules. Combinations of CCr and taxol were synergistically cytotoxic to breast tumor cells, indicating that CCr may be a useful addition to current cancer chemotherapy's that include taxanes.

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