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INHIBITION OF CCRF-CEM HUMAN LEUKEMIC LYMPHOBLASTS BY TRICIRIBINE (TRICYCLIC NUCLEOSIDE, TCN, NSC-154020)*

ACCUMULATION OF DRUG IN CELLS AND COMPARISON OF EFFECTS ON VIABILITY, PROTEIN SYNTHESIS AND PURINE SYNTHESIS

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Abstract—The experimental antineoplastic agent triciribine (tricyclic nucleoside, TCN) is known to be activated to its phosphate TCN-P by adenosine kinase and to inhibit cell growth, purine nucleotide synthesis, and incorporation of amino acids into proteins. Our objective in this paper was to compare these effects in intact cells of a human cell line as a prerequisite to describing in a companion paper [Moore *et al.*, *Biochem. Pharmac.* **38**, 4045 (1989)] more detailed enzymic studies of their interrelationships. TCN treatment inhibited cloning of CCRF-CEM human leukemic lymphoblasts 50% at concentrations of 6, 30, and 90 μM with 8-day, 8-hr, and 2-hr exposures respectively. However, 6–20% of the cells survived exposure to 200 μM TCN for 24 hr. The intracellular formation of TCN-P from TCN was rapid, concentrative and essentially complete, but TCN-P did not exceed about 1.4 mM (1.4 nmol/10^6 cells) at 200 μM TCN. In cells exposed to 50 μM TCN for 1.25 to 24 hr, formate incorporation into ATP and GTP was inhibited the most rapidly and strongly; pools of ATP and GTP were decreased as much as 40% (as compared with controls); and incorporation of formate into RNA purines was inhibited as much as 65%. Incorporation of leucine into protein was more moderately inhibited up to 40%, apparently in proportion to the concentration of intracellular TCN-P, rather than of the TCN in the medium. These inhibitions occurred most rapidly during the first 2–4 hr and increased only gradually thereafter, whereas cloning ability was inhibited more slowly and uniformly over a longer time period. No one of these metabolic effects by itself showed a clear correlation with the loss of viability. The incorporation of formate into formylglycinamide ribotide (FGAR, when accumulated at a blockade by azaserine) was inhibited drastically by TCN. The rate of incorporation of hypoxanthine into ATP was increased by TCN, whereas incorporation into GTP was decreased. Thus, the principal sites of inhibition of purine synthesis by TCN-P were shown in these intact cells to be at a step prior to synthesis of FGAR in the *de novo* pathway and also at an additional site between IMP and GTP.

Triciribine (also called tricyclic nucleoside, TCN‖; NSC-154020), in its more soluble prodrug form TCN-phosphate (TCN-P; triciribine phosphate, NSC-280594, Fig. 1), is an investigational antineoplastic agent. It has been used in phase 1 clinical investigations [1–4] and is now undergoing further tests. TCN was first synthesized by Schram and Townsend [5]. Early studies by Bennett *et al.* [6] and by Plagemann [7] revealed that it is phosphorylated by adenosine kinase to TCN-P but apparently undergoes no further phosphorylation. Both groups found that incorporations of precursors into DNA, RNA, and protein are inhibited to roughly similar degrees.

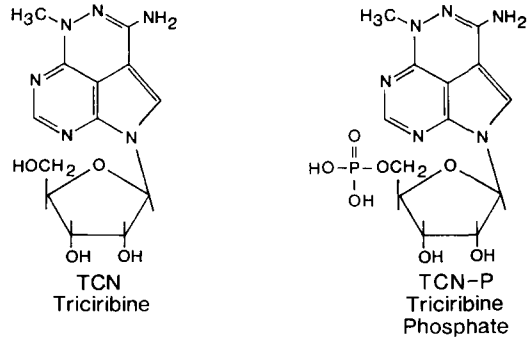

Fig. 1. Structures of TCN and TCN-P.

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‖ Abbreviations: TCN, tricyclic nucleoside, 6-amino-4-methyl-8-(β-D-ribofuranosyl)pyrrolo[4,3,2-de]-pyrimido-[4,5-c]-pyridazine, or 1-(β-D-ribofuranosyl)-3-amino-1,5-dihydro-5-methyl-1,4,5(6,8-pentaazaacenaphthylene, triciribine (NSC-154020); TCN-P, the 5′-phosphate of TCN (NSC-280594); PBS, phosphate-buffered saline (0.14 M NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 3 mM KCl, pH 7.5); and FGAR, formylglycinamide ribotide, 5-phospho-β-D-ribofuranosyl-αN-formylglycinamide.

Both Plagemann and Bennett *et al.* postulated an inhibition of *de novo* purine synthesis.

Schweinsberg [8] reported that TCN inhibits the incorporation of leucine and thymidine in CHO cells more than the incorporation of uridine, and that TCN-P inhibits *in vitro* incorporation of leucine by reticulocyte lysate. He also found that DNA polymerase partially purified from rat tumor is not inhibited by TCN-P [8]. Wotring *et al.* [9] reported that TCN-P is converted rapidly to TCN in human plasma and so serves as a prodrug. Cells lacking adenosine kinase are not inhibited by TCN-P. These authors also reported the metabolic effects of TCN on L1210 cells [10]. They found that, at the lowest effective doses of TCN, the cells are prevented from entering the S phase from G_1.

We report here our studies of the metabolic effects of TCN on intact human cells in culture, the leukemic lymphoblast cell line CCRF-CEM. The immediate purpose was to compare the relative degrees of a number of parameters: the amounts of TCN converted to TCN-P, the concentrations of the latter in cells, the inhibition of cloning of the cells, the inhibition of protein synthesis, and the inhibition of purine synthesis. The ultimate objective was to determine the mechanism(s) of inhibition of cell growth by this potentially useful drug. One of our longer range purposes has been to explore in detail the interesting problem of a drug which appears to inhibit the two separate processes, biosynthesis of proteins and biosynthesis of purine nucleotides. These studies were the background for additional studies on reproducing the inhibitions with cell-free systems, which will be reported separately [11]. Preliminary reports have already appeared [12, 13]. Initially we believed that the previously observed decay of ability to incorporate precursors into RNA and DNA could be consequences of the inhibitions of protein and nucleotide synthesis rather than primary effects. Therefore, in this work, we did not systematically evaluate polynucleotide synthesis to determine whether direct effects on RNA and DNA synthesis occurred, but by no means do we preclude such a possibility.

MATERIALS AND METHODS

CCRF-CEM leukemic lymphoblasts were obtained from William Plunkett, Ph.D., of this institution and were maintained in suspension culture in RPMI 1640 medium, from either Irvine Scientific (Santa Ana, CA) or Gibco (Grand Island, NY), plus 5% fetal calf serum (Hazelton, Denver, PA). They were counted in a model ZDI Coulter counter. Cell volume was determined using the same instrument calibrated with 10 μm latex beads.

TCN was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. For administration to cell cultures, TCN was dissolved in 0.1 N HCl, diluted in either medium or phosphate-buffered saline (PBS), and sterilized by filtration. Alamine-336® (tricapryl tertiary amine) was obtained from the Henkel Corp., Kankakee, IL. Hydrofluor scintillation fluid was from National Diagnostic, Somerville, NJ. HPLC columns were manufactured by Whatman (Clifton, NJ).

L-[4,5-^{3}H]Leucine (sp. act. 5 Ci/mmol), L-[2,3-^{3}H]arginine (sp. act. 17 Ci/mmol), [^{14}C]formate (sp. act. 55 mCi/mmol), [1-^{14}C]glycine (sp. act. 53 mCi/mmol), and [8-^{14}C]hypoxanthine (sp. act. 49 mCi/mmol) were obtained from New England Nuclear (Boston, MA).

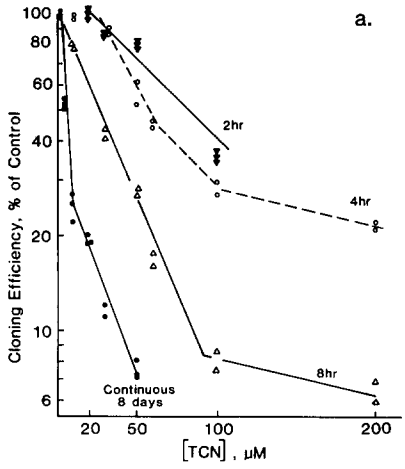
Cell cloning assays were done in methyl cellulose medium [14]. For timed exposure, known number of cells were exposed to TCN in liquid medium for a specified time and then centrifuged and resuspended in the cloning medium without drug. After incubation for 7–10 days, the colonies were fixed in 36% formaldehyde for 10 min, stained with 0.1% crystal violet and 50% ethanol, and counted.

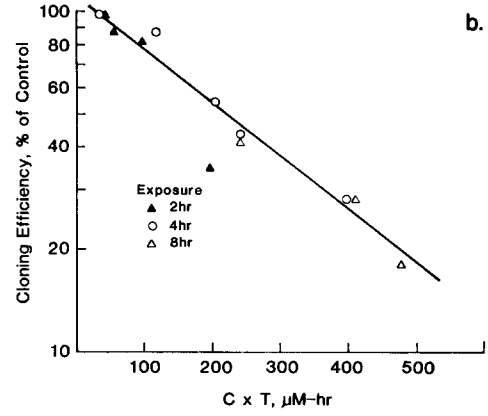
For isotope incorporation experiments, cells were exposed to TCN, and then labelled substrates (usually 1 μCi of each substrate per ml) were added and allowed to be incorporated for the last 45 min of the incubation period, which ranged from 75 min to 24 hr. Cells were counted, centrifuged, washed once in cold PBS, and then extracted with cold 0.4 N $HClO_4$ to obtain cold acid-soluble nucleotides and TCN metabolites.

RNA was extracted by 1 M NaOH at 37° overnight followed by precipitation of protein and DNA with 0.2 N $HClO_4$. Alternatively, mixed RNA and DNA were extracted by the hot potassium acetate procedure [15]. The residual protein fraction was processed by two washes (discarded) of the extracted residues with 0.4 N $HClO_4$ at 95–100° [15]. The label content of these fractions was determined in Hydrofluor-aqueous phase mixtures (5:1, v/v) with a Beckman LS-100 scintillation counter, using the external standard method of quench correction. When acid-soluble nucleotides were to be chromatographed, the cold $HClO_4$ supernatant fraction was neutralized by extraction with Alamine® in Freon [16].

Nucleotides were separated by high-pressure liquid chromatography (HPLC) on a Partisil 10 SAX column eluted with 0.005 M ammonium phosphate buffer (pH 3.0) for 10 fractions, and then with a linear gradient of ammonium phosphate buffer (pH 3.0), 0.005 to 0.75 M, for 70 fractions. The flow rate was 2.0 ml/min, and the fraction size was 2.0 ml. The effluent was monitored by ultraviolet absorbancy at 260 and 280 nm, which was recorded on a dual pen recorder. Peak areas were measured by planimetry. The quantities of nucleotides and TCN metabolites were calculated using constants derived by chromatography of spectrophotometrically standardized samples. Intracellular concentrations were calculated using the nucleotide or metabolite quantity, the number of cells, the aliquot factors, and the cell volume (about 1 μl/10^6 cells, determined separately). Incorporation of radioactivity was determined by counting an aliquot of each fraction in the nucleotide peak.

TCN-P (Fig. 1) was measured by combining fractions 5–8 from the Partisil SAX column (fractions 2–4 contained the TCN) and rechromatographing a 2-ml aliquot on a Partisil 10 ODS-2 column with an 80-ml linear gradient from 0.1 M ammonium phosphate (pH 6.0) to the same buffer containing 30% meth-





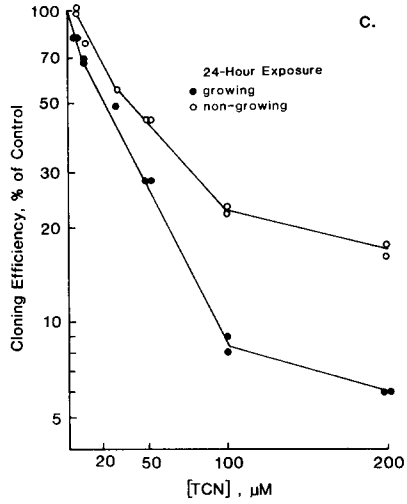


Fig. 2. Effect of TCN concentration and treatment time on cloning efficiency of CCRF-CEM cells. Panel a: Cells were exposed to TCN at the concentrations and for the times indicated and then washed and plated in methylcellulose medium (except for the continuous exposure group). Continuous exposure cells were plated in methylcellulose medium containing TCN. Clones were stained and counted after 8 days. Each point is the average of triplicate samples. The 4- and 8-hr plots were determined in the same experiment, the others each in a separate experiment. The plating efficiency of the controls was 88% for the 2-hr experiment, 98% for the 4- and 8-hr experiment, and 101% for the continuous exposure experiment. Panel b: Survival is plotted against the product of concentration × time for the experiments of Panel a at times up to 8 hr. The data for 24 hr and 8 days fell above the line, as did the 8 hr, 200 μM point. Panel c: Initially growing (0.1×10^6/ml) and non-growing (1.2×10^6/ml) cells were exposed to TCN for 24 hr, and the plating efficiency was determined. The efficiency of both controls was 100%. Each point is the average of duplicates.

anol (v/v) [see Ref. 17]. Flow rate was 1.0 ml/min, and fraction size was 1.0 ml. The effluent was monitored at 280 nm. TCN-P emerged in fractions 33–39; the TCN peak appeared at about fraction 70. In some cases the 8 ml collected from the SAX column was dried and dissolved in a smaller volume of water before the entire sample was applied to the ODS column. TCN and TCN-P solutions were standardized spectrophotometrically, assuming the millimolar absorbancy at 292 nm in 0.1 M NaOH to be 11.7.

RESULTS

Loss of viability. The results of cloning assays on TCN-treated cells are shown in Fig. 2a. With continuous exposure, half the cells were killed by about 6 μM TCN; with shorter exposures of 8, 4 and 2 hr, higher concentrations of 30, 70 and 90 μM, respectively, were required to kill 50% of the cells. Figure 2b shows a composite replot of the lower range of dose–time products (μM concentration × exposure time in hr) versus log survival. Only for concentrations up to 100 μM and times up to 8 hr did the data fall approximately on a line, showing 50% survival at about 230 μM · hr. At exposures greater than this, the linearity of the survival plots decreased and the slopes increased, indicating increased resistance. About 6–8% of the cells were resistant to the longest times (8 days at 50 μM) or highest concentrations (200 μM for 8 hr) tested. Experiments to compare cells under log- and plateau-growth conditions (Fig. 2c) showed that the slower growing cells were markedly more resistant. Thus, the exact degree of cell killing can vary with the initial state of the cells.

Intracellular TCN-P. The phosphorylation of TCN was rapid but limited (Fig. 3). The concentration of TCN-P attained after exposure to 10 μM TCN for 0.5 hr was about 0.4 mM, whereas that with 200 μM TCN for 1.25 to 12 hr was only 1.4 mM; the maximum was reached by 2 hr. With 5 μM TCN in the medium, a 100-fold concentration factor was seen. The intracellular TCN amounts were minor compared to TCN-P amounts and were judged due primarily to incomplete washing of cells. The loss of TCN-P was also rapid (Fig. 3b); the half-life was less than 1 hr after drug removal.

Degrees of inhibition of protein and purine synthesis. In short-term experiments with logarithmically growing cells (75 min with drug including 45 min with labelled precursor), the incorporation of

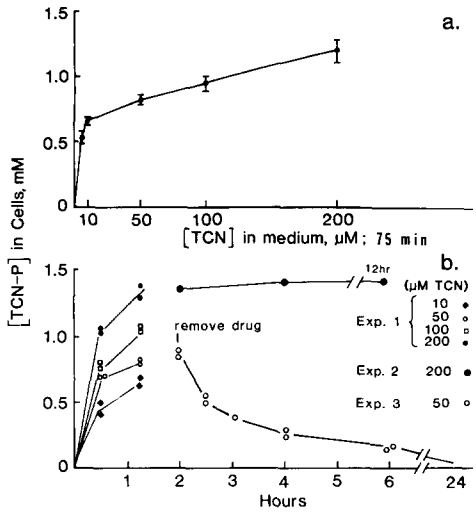


Fig. 3. Accumulation and loss of TCN-P in cells. Panel a: Intracellular TCN-P accumulation in log-phase cells exposed to various concentrations of TCN for 75 min is shown with the average and standard errors for three experiments. Panel b: Intracellular TCN-P concentration is plotted against time. Three experiments are shown. In experiment 1, log phase cells were exposed to various concentrations for 30 and 75 min. In experiment 2, the cells, initially in log phase, were exposed to 200 μM TCN for 2, 4 and 12 hr. The controls reached the end of log phase; the treated cells grew slightly and then declined. In experiment 3, after exposure to 50 μM TCN for 2 hr, the drug was removed and disappearance of TCN-P followed. The cells resumed growth by 4 hr but did not reach plateau phase. TCN-P was measured chromatographically in extracts of cells washed with PBS. Since the average cell volume was 1 μl/10^6 cells, 1 nmol/10^6 cells equalled 1 mM for normal-size cells.

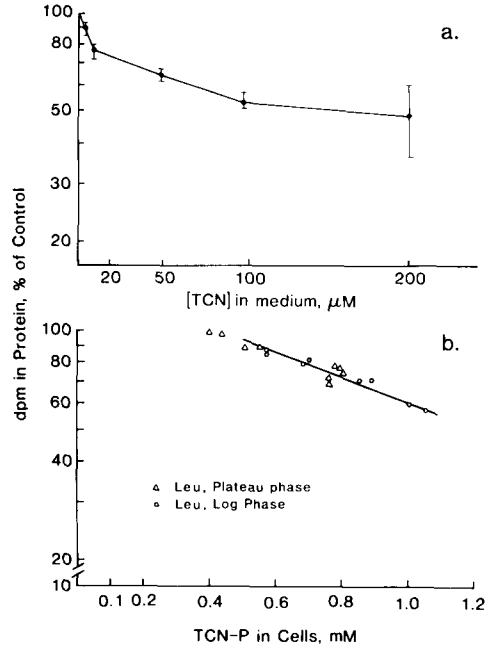


Fig. 4. Effect of several concentrations of TCN on incorporation of [^{3}H]leucine into protein. Panel a: Data from six experiments with [^{3}H]leucine and one with [^{3}H]arginine in log-phase cells are summarized. Cells were exposed to TCN for 75 min including 45 min with labeled amino acid. The control incorporation for leucine was 10.5 ($\pm$1.3) $\times$ 10^3 dpm/10^6 cells (mean $\pm$ SD). Panel b: Incorporation of [^{3}H]leucine is plotted against the measured intracellular TCN-P concentration in log-phase (0.65 $\times$ 10^6 cells/ml) and non-growing (1.3 $\times$ 10^6 cells/ml) cells. The controls incorporated 8.9 and 8.7 $\times$ 10^3 dpm/10^6 cells. Each point is the average of triplicates (one experiment). The TCN concentrations were 5, 10, 50, and 100 μM.

leucine and arginine were inhibited about 25% at 10 μM TCN. The inhibition leveled off at 50% or less with 100 or 200 μM TCN (Fig. 4a). Some inconsistencies in the degrees of inhibition were traced to variation in cell densities. In three experiments at 100 μM TCN, plateau phase cells (over 10^6/ml) were inhibited only an average of 24 $\pm$ 5% (SD), whereas log phase cells (under 7 $\times$ 10^5/ml) were inhibited an average of 47 $\pm$ 5% (data not shown); the difference was significant at the 0.05 level. However, when leucine incorporations were plotted against the measured intracellular TCN-P concentration (Fig. 4b), the points for both log- and plateau-phase cells fell on the same line. Thus, in these short-term experiments, the difference in inhibition was due to the greater accumulation of TCN-P by the log phase cells.

TCN inhibited incorporation of [^{14}C]formate or glycine into nucleic acid purines more than it inhibited incorporation of [^{3}H]leucine into protein, but with a similar pattern. This differential inhibition was observed in four experiments, each using both labeled substrates, including eight comparisons with triplicate samples. Five comparisons showed greater inhibition of incorporation into RNA than of incorporation into protein in the same cells, one (at 10 μM

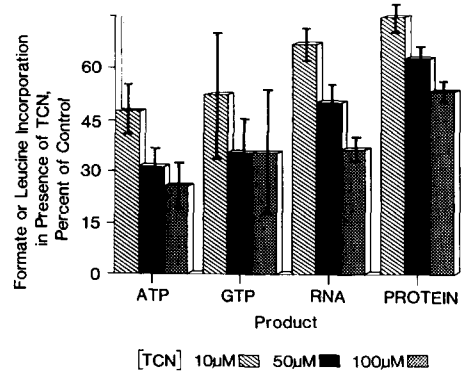


Fig. 5. Comparison of effects of exposure to TCN on synthesis of purine nucleotides, RNA, and proteins. The effects on incorporation of [^{14}C]formate into cold perchloric acid-soluble ATP and GTP, and into RNA, are compared with those on incorporation of [^{3}H]leucine into protein. Several experiments with log-phase cells (0.56–0.9 $\times$ 10^6 cells/ml) incubated for 75–120 min with drug including a final labeling period of 45 min are summarized. The mean and standard error of three to six experiments, each with duplicate or triplicate samples, are shown. The average control value for leucine incorporation was 10,500 $\pm$ 1300 dpm/10^6 cells. The average control value for formate in RNA was 3300 $\pm$ 1600 dpm/10^6 cells. For formate in ATP and GTP (four experiments), the range of controls was 900 to 7900 and 600 to 6300 respectively.

TCN) showed the reverse, and two showed equal inhibition.

Figure 5 compares the average incorporation into RNA, ATP, GTP and protein at three concentrations of TCN in these and other short-term experiments. At each concentration the average inhibition of incorporation into protein was less than that of incorporaltion into purines and RNA. At 100 μM TCN, incorporation into RNA was inhibited 63 ± 3% (SE), and into protein only 46 ± 3%; the difference was significant at the 0.05 level.

The incorporation of formate into the RNA precursor pools, ATP and GTP, was usually inhibited to a somewhat greater degree than was incorporation into nucleic acids in the same experiment. The average inhibition of incorporation into ATP by 100 μM TCN was 74 ± 7%. The relative effects on GTP synthesis were markedly more variable than the other effects.

The formate-labeling pattern was qualitatively similar whether the material counted consisted of total cold perchloric acid-insoluble fraction, total nucleic acids, or alkali-hydrolyzed RNA. Similar results were obtained in a single experiment using [^{3}H]glycine as precursor for RNA; these are included in the averages. Because these experiments were designed to study inhibition of purine synthesis, and because labeling of DNA by formate would include label in thymidine as well as purines, we did not attempt to measure separately incorporation into DNA, which would be minimal in this short time period.

Time course of inhibition of protein and RNA synthesis. The time course was studied over exposure periods of 2–24 hr with 10, 20 and 50 μM TCN. This was a single experiment in which many parameters were studied in the same batch of cells. (A similar experiment with fewer time points gave similar

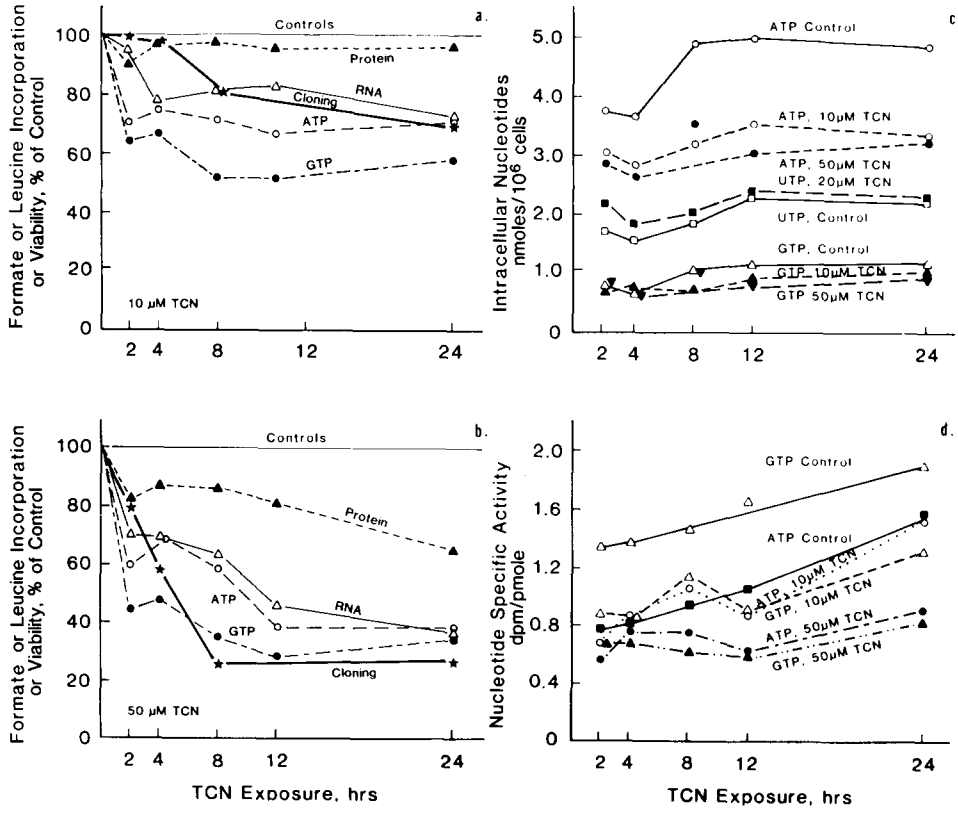


Fig. 6. Time course comparison of effects of TCN. Labeled substrates were added for the last 45 min in each case. The cell concentration was 0.2×10^6 cells/ml at the beginning; the controls reached 0.4×10^6 at 24 hr, which is still log phase. This is a single experiment in which all four parameters were measured in the same cells at 0, 10 and 50 μM TCN. Panel a: Incorporation of [^{3}H]leucine into protein and [^{14}C]formate into cold perchloric acid-soluble ATP and GTP and into RNA (dpm per 10^6 cells), expressing the average of duplicates as a percentage of control values. TCN concentration in the medium was 10 μM. Panel b: Same as Panel a, except that TCN in the medium was 50 μM. Control values (10^3 dpm/10^6 cells) for Panels a and b are as follows: Leu: 2 hr, 11.60; 4 hr, 14.90; 8 hr, 17.70; 12 hr, 24.09; and 24 hr, 21.35. RNA: 2 hr, 1.98; 4 hr, 2.80; 8 hr, 3.35; 12 hr, 3.27; and 24 hr, 6.39. ATP: 2 hr, 2.60; 4 hr, 2.90; 8 hr, 4.09; 12 hr, 4.26; and 24 hr, 6.82. GTP: 2 hr, 0.91; 4 hr, 0.88; 8 hr, 1.28; 12 hr, 1.41; and 24 hr, 2.28. Panel c: Nucleotide pool sizes, nmol per 10^6 cells, in the same experiment as in Panels a and b. Panel d: Nucleotide specific activities, dpm per pmol, in the same experiment as Panels a, b and c.

results; data not shown.) The cells were diluted with fresh medium in replicate flasks about 24 hr before TCN was added to the experimental flasks at zero time. Prior to each time point, the cells were labeled for 45 min with [^{14}C]formate and [^{3}H]leucine simultaneously. The results are shown in Fig. 6. (It should be noted that this experiment showed less inhibition by TCN than we have usually seen—compare with Fig. 5.) For simplicity, only the results with 10 and 50 μM TCN are shown. The results of the cloning assay from Fig. 2 are also plotted for comparison. During the course of the experiment, the cell numbers in the controls remained constant through the first 8 hr, increased about 18% at 12 hr, and reached 177% of the initial number at 24 hr. The cell numbers in the TCN-treated flasks were the same as those in the controls through the first 12 hr, but were slightly lower (150% of original) at 24 hr with 50 μM TCN.

From the cloning results with 10 μM TCN, we expect over 95 and 70% of the cells to remain viable for the 2- and 12-hr periods, respectively, while at 50 μM about 80 and 27% remain viable for these periods. In contrast to these slower-developing effects on cloning, many of the inhibitory effects on synthesis were exhibited in the first 2 hr. The inhibition of protein synthesis by 10 μM TCN (Fig. 6a) was minimal in this experiment, within the range of variation of the controls. With 50 μM TCN (Fig. 6b), the inhibition increased only slightly between 2 and 24 hr to a maximum of 30%. It is not clear whether the apparent recovery of protein synthesis between 2 and 4 hr is reproducible; another experiment with 200 μM TCN showed protein synthesis to be 51 and 53% of control at 2 and 4 hr respectively. Compared to protein synthesis, inhibition of formate incorporation into RNA purines was more severe, but not complete; it increased with time when 50 μM TCN was used, but at a diminishing rate after 2 hr, and reached a maximum of 60% inhibition at 24 hr.

Time course of inhibition of nucleotide synthesis. In the *control* cells, the average amount of ATP per cell increased 29% and that of GTP 49% by 8 hr (during this period the cell number did not change) and remained almost constant thereafter, as shown in Fig. 6c. The ratio ADT/ATP was about 0.1 (not shown). Figure 6d shows that the specific activities of ATP and GTP in the *controls* increased linearly with time up to 24 hr.

* We thank Dr W. Plunkett for assistance with this analysis.

In *treated*, compared to control cells, the effect of TCN on ATP was an immediate large decrease in the *rate* of *de novo* synthesis from formate (Fig. 6, a and b). The *pool size* of ATP (Fig. 6c) was decreased about 20% at 2 hr with both 10 and 50 μM TCN, and with the exception of one point, it fluctuated thereafter between 59 and 75% of the controls. The *specific activity* (Fig. 6d) was also decreased, especially at the 50 μM TCN level. TCN had an even greater effect in decreasing the rate of synthesis of GTP, but a lesser effect in decreasing the pool size; hence, it had a great effect in depressing the specific activity of GTP. The net result was that ATP and GTP acquired the same specific activity which was depressed markedly compared to the controls. These data indicate that TCN inhibits *de novo* synthesis of both ATP and GTP.

Lack of effects on other nucleotides. The UTP (Fig. 6c) and CTP (not shown) pool sizes were *not* decreased in inhibited compared to control cells. They were slightly greater at most time points (maximum increase of 30% only at the 2-hr point), a pattern observed in other experiments. The ADP/ATP ratios also were not changed significantly by TCN. In a separate experiment with large batches of cells, one sample for each treatment, pools of deoxynucleotides in cells treated for 2 hr with 100 μM TCN differed by less than 12% from those of the control cells (data not shown). Deoxynucleotides were determined chromatographically after removal of ribonucleotides with periodate [18].*

Sites of purine inhibition. Table 1 shows that incorporation of [^{14}C]hypoxanthine into total acid-soluble purine nucleotides was increased 19% by treatment with 100 μM TCN. A differential effect on adenine and guanine nucleotides was observed; incorporation of label into adenine nucleotides increased by 32%, but incorporation into guanine nucleotides decreased by 23%. Decreases in the nucleotide pool sizes in the presence of TCN were relatively small; thus, the ratio of the specific activity of guanine over that of adenine in the acid-soluble nucleotides changed from 0.95 in the controls to 0.57 in the cells treated with 100 μM TCN for 2 hr.

To help determine the locus of TCN effects in purine synthesis, we measured its effect on intracellular synthesis of the intermediate formylglycinamide ribotide (FGAR). Accumulation of FGAR can be induced by blocking the next step in the pathway (amidation of FGAR) with the glutamine analog azaserine, as previously described [19, 20].

Table 1. Effect of TCN on incorporation of hypoxanthine*

TCN in medium (μM)	ATP + ADP			GTP + GDP			Ratio
	Amount (nmol)	dpm ($\times 10^{-3}$)	Sp. act. (dpm/pmol)	Amount (nmol)	dpm ($\times 10^{-3}$)	Sp. act. (dpm/pmol)	Sp. act. G/A
0	5.5	177	32.2	1.41	43	30.5	0.95
10	5.0	188	36.2	1.23	41	33.3	0.92
50	5.3	226	42.6	1.28	36	28.1	0.66
100	5.1	233	45.7	1.26	33	26.2	0.57

* Cells were exposed to TCN for 2 hr; the last 45 min included exposure to [^{14}C]hypoxanthine. Each value is the average of duplicate samples, and the amounts are expressed per 10^6 cells.

Table 2. Effect of TCN on early steps of purine biosynthesis*

Drug added (μM)	Incorporation of [^{14}C]formate per 10^6 cells					
	ATP		GTP		FGAR	
	(dpm)	(%)	(dpm)	(%)	(dpm)	(%)
None	3933	100	1127	100	0	0
TCN (200)	830	21	232	21	0	0
Azaserine (300)	109	3	24	2	22,355	100
TCN + Azaserine	65	2	16	1	3,860	17

* Cells were exposed to TCN and/or azaserine for 2 hr, and then [^{14}C]formate was added for 45 min. ATP, GTP, and FGAR in the acid-soluble extract were separated by HPLC. FGAR was identified as the only labeled peak on the chromatogram which was derived from azaserine-treated cells but not from control cells; it was separately shown to be labeled by [^{14}C]glycine as well as by formate.

Table 2 shows that, as expected, azaserine alone blocked incorporation of [^{14}C]formate into ATP and GTP, and caused a large accumulation of label in FGAR. It further shows that TCN plus azaserine blocked most of this accumulation. The degree of inhibition of FGAR accumulation by TCN (83%) was almost identical with its inhibition of ATP synthesis (79%) in the absence of azaserine. Thus, TCN directly inhibited formation of FGAR or one of its precursors, glycinamide ribotide, phosphoribosylamine or phosphoribosyl pyrophosphate. Effects on the latter, PRPP, were judged unlikely because no clear effects on pyrimidine nucleotide concentrations were observed.

DISCUSSION

Half or more of the human CCRF-CEM cells were killed by continuous exposure to 6 μM TCN, and 90% by about 40 μM TCN. With 24-hr exposure, about 40 μM was required to kill 50% of the cells. The CCRF-CEM cells are much less sensitive than L1210 cells (75% killing by 1 μM TCN in 24 hr [10]) or Novikoff cells (destroyed by 5–15 μM TCN within 3 hr [7]). They are perhaps more sensitive than CHO cells (which require 20 μM to suppress cloning and survive at 15 μM [8]). They are much more sensitive than HeLa cells (growth suppressed and some cells killed within 48 hr by 200 μM TCN, although apparently not killed by 120 μM for 80 hr [7]). HEp-2 cells are even less sensitive than HeLa cells [7]. Thus, CCRF-CEM cells are within a wide range of sensitivities.

A large fraction of the cells was able to survive high concentrations of TCN for 24 hr: 6% of cells initially in log growth and 18% of cells in plateau phase. This diminished response is probably the result of two factors: the actual intracellular concentration of TCN-P and the resistance of certain cell populations or possibly of certain phases of the cell cycle. In the experiment shown in Fig. 4b, the reduced inhibition of protein synthesis was explained by a smaller accumulation of TCN-P in the more crowded cells; here the exposure times were short (75 min). We have no direct evidence that the observed resistance of non-growing cells in Fig. 2c is a cell cycle specific difference in sensitivity to TCN-P; however, Wotring *et al.* [10] have reported that TCN causes accumulation of L1210 cells at the G_1-S boundary and that TCN is lethal to cells in S phase.

TCN-P was formed rapidly from low TCN levels in the medium, and accumulated in these cells. The accumulation of TCN-P was limited to about 1.5 mM and diminished rapidly when the TCN was removed from the medium. This seems unlike the observations of Zhengang *et al.* [21] and Schilcher *et al.* [4], who found TCN-P in tissues as long as 6 and 8 weeks after treatment, and Lu *et al.* [22] and Powis *et al.* [1], who found a half-life of about 90 hr for TCN-P in blood cells. The difference could be explained by the efficient phosphorylation of TCN, the long half-life of TCN in blood [1, 4, 22], and its recycling through bile and gut [4, 23]. The level of TCN found in plasma [1, 4, 22], less than 0.6 μg/ml (1.7 μM), however, would not have been high enough to kill a significant fraction of CCRF-CEM cells.

We had originally hoped that the dose and time relationships of one or more of these inhibitions would correlate with those of cell killing in the cloning assays and so offer a clue as to which effect of TCN was most lethal. This hope was not fulfilled (compare Fig. 2a with Figs. 4a and 5, and cloning line with others in Fig. 6a and 6b). Loss of viability appears to increase more with dose, and especially with time, than any of the inhibitions we have studied.

The maximum observed decreases in purine nucleotide pools (about 40%) hardly seem sufficient to be lethal. Earlier investigators [6–8] have indicated that lack of purines is not the cause of cytotoxicity, because addition of purine metabolites did not reverse TCN toxicity, although they did not establish that nucleotide pools were restored to normal by the additions.

Although inhibition of DNA synthesis is usually coupled to long-term inhibition of protein synthesis [24], the degree of such inhibition of these CCRF-CEM cells (a maximum of about 50%) does not seem adequate for lethality. Other investigators of TCN action have reported roughly similar large inhibitions of protein and DNA synthesis [6, 7], but the literature does not, at present, provide enough detailed studies to permit a conclusion as to whether TCN has a direct primary action on DNA replication.

The question whether the inhibition of RNA synthesis by TCN was a direct effect (rather than a reflection of decreased labeling of purine nucleotides) was examined. Calculations (not shown) using the data of Fig. 6 provided *relative* values comparing the amounts of ribonucleotides incorporated into RNA. The values were sufficiently similar for all doses and times to suggest that little if any direct inhibitory effect on the transcription step was involved in *short-term* labeling by formate. Thus, the apparent inhibition of RNA synthesis in these short-term experiments was due primarily to the decrease in the specific activity of the nucleotides.

The data presented here suggest but do not estab-

lish that inhibition of protein synthesis is independent of the inhibition of purine synthesis. Subsequent work with cell-free systems for incorporation of amino acids into proteins [13, 25, *] has demonstrated, however, significant direct effects of TCN-P on amino acid acylation.

The increased labeling of adenine nucleotides by hypoxanthine and the decreased labeling by glycine and formate indicate that TCN inhibits *de novo* purine synthesis prior to the formation of IMP. Bennett *et al.* [6] observed similar effects on incorporation of formate and hypoxanthine and suggested a similar conclusion. Consideration of these data together with the decrease in labeling of FGAR led to the conclusion that a primary site of TCN (TCN-P) action is one or more of the early steps in *de novo* purine synthesis, up to or including FGAR synthetase. We have further studied the site of this inhibition by use of cell-free extracts with TCN-P, and have localized it to the amidophosphoribosyl transferase step, as detailed separately in the accompanying paper [11].

The decreased incorporation of hypoxanthine into guanine compared to the increased incorporation into adenine nucleotides (Table 1) indicate that TCN also effects the conversion of IMP to guanine nucleotides. In conjunction with the similar differential inhibition of labeling of ATP and GTP by *formate* (see Fig. 6), these data suggest an additional separate site of inhibition by TCN (TCN-P) at the level of IMP–GMP–AMP interconversions. In the simplest case, this could be at the IMP dehydrogenase or XMP aminase steps, more likely the former because no accumulation of XMP was observed chromatographically. This prediction has been confirmed enzymatically [11].

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REFERENCES

1. G. Powis, P. J. Basseches, D. M. Kroschel, R. L. Richardson, M. J. O'Connell and L. K. Kvols, *Cancer Treat. Rep.* **70**, 359 (1986).
2. L. G. Feun, N. Savaraj, G. P. Bodey, K. Lu, B. S. Yap, J. A. Ajani, M. A. Burgess, R. S. Benjamin, E. McKelvey and I. Krakoff, *Cancer Res.* **44**, 3608 (1984).
3. A. Mittleman, E. S. Casper, T. A. Godwin, C. Cassidy and C. W. Young, *Cancer Treat. Rep.* **67**, 159 (1983).
4. R. B. Schilcher, C. D. Haas, M. K. Samson, J. D. Young and L. H. Baker, *Cancer Res.* **46**, 3147 (1986).
5. K. H. Schram and L. B. Townsend, *Tetrahedron Lett.* **49**, 4757 (1971).
6. L. L. Bennett, Jr., D. Smithers, D. L. Hill, L. M. Rose and J. A. Alexander, *Biochem. Pharmac.* **27**, 233 (1978).
7. P. G. W. Plagemann, *J. Natn. Cancer Inst.* **57**, 1283 (1976).
8. P. D. Schweinsberg, *Ph.D. Dissertation*, University of Texas Graduate School of Biomedical Sciences at Houston (1981).
9. L. L. Wotring, G. W. Crabtree, N. L. Edwards, R. E. Parks, Jr and L. B. Townsend, *Cancer Treat. Rep.* **70**, 491 (1986).
10. L. L. Wotring, J. E. Passiatore, J. L. Roti Roti, J. L. Hudson and L. B. Townsend, *Cancer Res.* **45**, 6355 (1985).
11. E. C. Moore, R. B. Hurlbert, G. R. Boss and S. P. Massia, *Biochem. Pharmacol.* **38**, 4045 (1989).
12. E. C. Moore and S. P. Massia, *Fedn Proc.* **44**, 681 (1985).
13. E. C. Moore, R. B. Hurlbert and S. Massia, *Proc. Am. Ass. Cancer Res.* **26**, 246 (1985).
14. L. W. Dow, D. E. Bell, L. Poulakos and A. Fridland, *Cancer Res.* **40**, 1405 (1980).
15. M. Schaffer, R. B. Hurlbert and A. Orengo, *Cancer Res.* **33**, 2265 (1973).
16. J. X. Khym, *Clin. Chem.* **21**, 1245 (1975).
17. P. D. Schweinsberg, R. G. Smith and T. L. Loo, *Biochem. Pharmac.* **30**, 2521 (1981).
18. B. A. Harris and W. Plunkett, *Cancer Res.* **41**, 1039 (1981).
19. J. F. Henderson, *Regulation of Purine Biosynthesis*, p. 166, American Chemical Society, Washington, D.C. (1972).
20. E. C. Moore and G. A. LePage, *Cancer Res.* **17**, 804 (1957).
21. G. Zhengang, N. Savaraj, K. Lu, L. G. Feun, M. A. Burgess, R. S. Benjamin and T. L. Loo, *Proc. Am. Soc. clin. Oncol.* **2**, 41 (1983).
22. K. Lu, N. Savaraj, L. G. Feun, R. S. Benjamin, J. P. Hester and T. L. Loo, *Proc. Am. Soc. clin. Oncol.* **2**, 34 (1983).
23. P. J. Basseches and G. Powis, *Cancer Res.* **44**, 3672 (1984).
24. E. Stimac, D. Houseman and J. A. Huberman, *J. molec. Biol.* **115**, 485 (1977).
25. E. C. Moore, R. B. Hurlbert, J. M. Ravel and S. P. Massia, *Proc. Am. Ass. Cancer Res.* **27**, 303 (1986).

* E. C. Moore, Inhibition by TCN-P of aminoacyl-t-RNA synthetases and *in vitro* protein synthesis, manuscript in preparation.