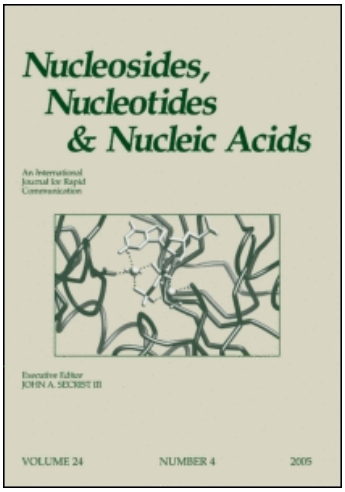

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Triciribine (TCN), A Novel Tricyclic Adenosine Analog with Anticancer Activity

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TRICIRIBINE (TCN), A NOVEL TRICYCLIC ADENOSINE ANALOG WITH ANTICANCER ACTIVITY

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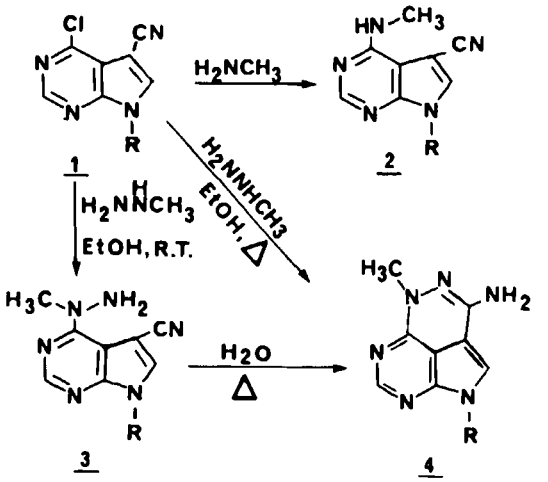
ABSTRACT. Triciribine shows promise as an antitumor drug and is currently in clinical trials. In L1210 cells *in vitro*, triciribine lethality is associated with inhibition of DNA synthesis, a G_1(-S) cell progression block and slowing of progression of cells through S phase.

TCN(6-amino-4-methyl-8-(ß-D-ribofuranosyl)pyrrolo[4,3,2-*de*]-pyrimido[4,5-*c*]pyridazine) is a novel adenosine analog which has antitumor activity *in vivo* and *in vitro*, and is currently in clinical trials. This paper will summarize the development of TCN as an antineoplastic drug and present some of our recent findings on its biochemical mechanisn of action.

TCN was synthesized as a nucleoside having a novel tricyclic ring structure[1]. A pyrrolo[2,3-*d*]pyrimidine was bridged across the top with a pyridazine ring by reacting 4-chloro-5-cyano-7-(ß-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine(1) with methyl hydrazine in ethanol (Scheme 1). The structure 4 was assigned on the basis of extensive pmr and ir spectroscopic studies, as well as chemical studies involving 2 and 3. The exocyclic amino group is in quite a different position than in adenosine, and the nitrogen atom analogous to the 6-NH_2 of adenosine has been incorporated into the pyridazine ring. Nevertheless, TCN functions biochemically in some respects as an



SCHEME 1

analog of adenosine. For example, it is phosphorylated by adenosine kinase.[2,3] However, it is not a substrate for adenosine deaminase,[4,5,6] nor is TCN monophosphate a substrate for AMP deaminase.[6] This resistance to deamination is, of course, advantageous from a chemotherapeutic point of view since it may provide a longer biological half life for the drug than what it might have if it were readily deaminated.

We have studied the cellular and biochemical effects of TCN in L1210 cells.[7] First, continuous treatment of the cells with TCN inhibited cell growth, with maximal growth inhibition being observed at 1 μM (FIG. 1). Prolonged incubation at this concentration killed a substantial fraction of the cells, about 80% after 24 hr, as shown by colony forming ability (FIG. 2). To obtain a preliminary indication of what biochemical effects might be involved in the lethal effect of TCN, we investigated the perturbations of cell cycle kinetics induced by treatment with TCN. The DNA content of individual cells, stained with propidium iodide, was determined by flow cytometry. Then the fractions of the population in the various cell cycle phases were derived from this data as described previously.[7] As shown in FIG. 3, continuous treatment with 1 μM TCN caused an accumulation of cells in the G_1 phase and a decrease in S phase,

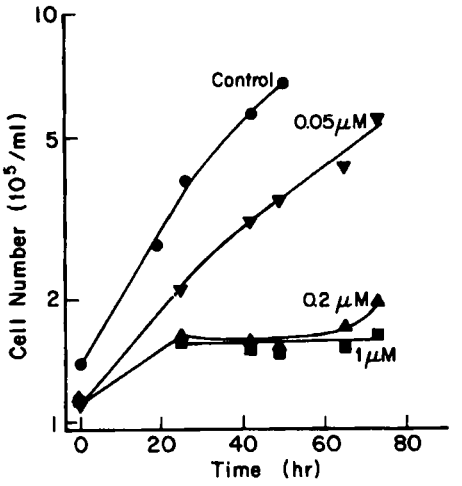


FIG. 1. Effect of various concentrations of TCN on growth of L1210 cells. ●, control; ■, 1 μM TCN; ▲, 0.2 μM TCN; ▼, 0.05 μM TCN. L1210 cells were grown in static suspension cultures at 38°C, in Fischer's medium for leukemic cells of mice, with 10% horse serum. Cell number was determined using a Coulter counter, on duplicate samples at each time point, and the average values plotted.

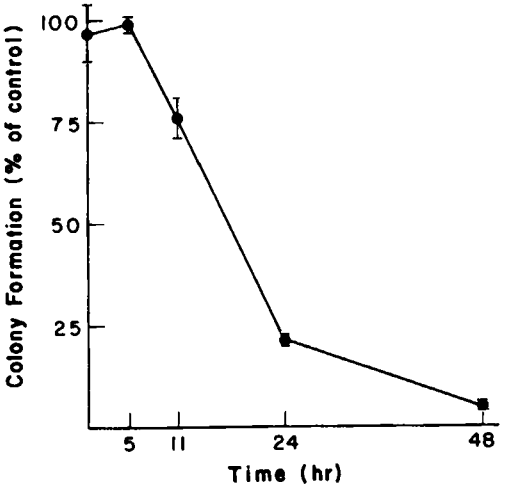


FIG. 2. Effect of 1 μM TCN on the viability of L1210 calls as determined by colony formation. After various times of TCN treatment the cells were separated from the TCN-containing medium by centrifugation, washed and resuspended in 0.13% Difco noble agar in Fischer's medium with 15% horse serum. The number of cells per tube was adjusted to provide approximately 100 colonies per tube. After 2 weeks incubation, the macroscopic colonies in each tube were counted using a dissecting microscope. Each determination consisted of 9 replicate tubes, and the values in the figure are the means of 3-6 determinations. Bars, SE. The absence of a bar indicates that the SE falls within the size of the point. Control colony formation was 57 ± 2% (SE).

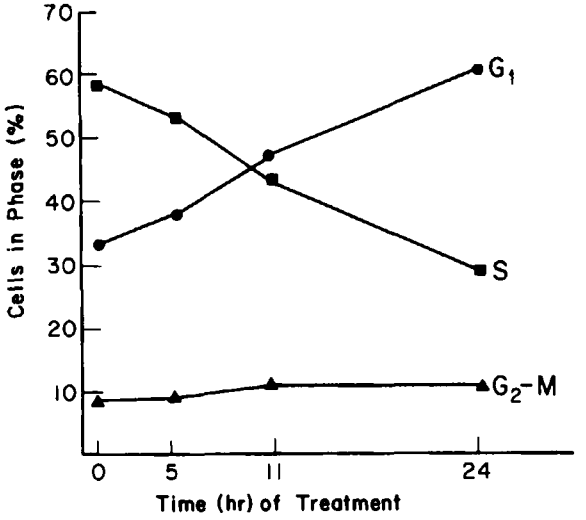


FIG. 3. Effect of 1 μM TCN on the cell cycle distribution of L1210 cells. After various times of TCN treatment, cells were stained with an acriflavine/Feulgen procedure and analyzed using a Becton-Dickinson FACS III flow cytometer as described previously.[7] The fractions of the cell population in G_1, S, and G_2-M were calculated by simulation analysis of the DNA histograms. The control cell cycle distribution remained constant throughout the experiment and is represented by the points at 0 time of TCN treatment. The values shown for TCN-treated cells are the averages of 2-5 independent experiments, and the control values (0 h) are the averages of 12 determinations. The SE was always less than 10% of the value, and typically less than 5% of the value, for the G_1- and S-phase fractions.

while the fraction in G_2-M remained essentially constant. These results indicated that TCN caused a block to cell progression in G_1 or at the G_1-S boundary. This conclusion was tested using G_1 cells selected by centrifugal elutriation (FIG. 4A and B) These G_1 cells were incubated as controls (FIG. 4C) or with 1 μM TCN (FIG. 4D) for 12 hours, during which the cells progressed into the cell cycle, but the TCN-treated cells remained in G_1. This result confirmed that TCN prevented G_1 cells from entering S phase.

Now, let us address the question, what happened to cells that were already in S phase when TCN was added to an exponentially growing culture? The decrease in the fraction of the population in S phase

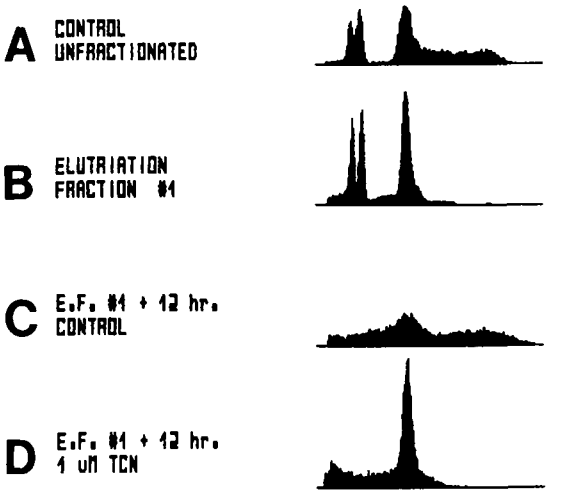


FIG. 4. Effect of TCN on progression of G_1 cells. Histograms of DNA content obtained by flow cytometric analysis of cells fixed with ethanol and stained with propidium iodide are shown. Ordinate, number of cells; abscissa, flourescence intensity, which is proportional to DNA content. Trout erythrocytes and human leukocytes, which correspond to the two peaks with the lowest DNA content, were used as DNA content markers in A and B. A. Exponentially growing L1210 cells. B. First fraction from centrifugal elutriation, containing predominantly G_1 cells. C. Cells from the first fraction incubated 12 hr as controls. D. Cells from the first fraction incubated 12 hr with 1 μM TCN. Centrifugal elutriation was performed using a Beckman J2-21M centrifuge with a JE-6B elutriation rotor. The speed was held constant at 2000 rpm, and 50-100 ml fractions were collected at incremental flow rates from 10-60 ml/min. The first (smallest size) fraction containing a significant number of cells (collected at approximately 25 ml/min) comprised 11-17% of the total number of cells (approximately 10^8) loaded into the elutriation chamber; 85-90% of the cells in this fraction were in G_1 phase, the remainder in S phase, as determined by flow cytometry and ^{3}H-dThd labeling.

(FIG. 3) could be accounted for by progression of cells out from behind the G_1(-S) block. However, this progression did not occur at the normal rate, as suggested by the decrease in the growth rate during the first 24 hours of TCN treatment (FIG. 1). This point was further demonstrated when the concentration of TCN was varied. When the concentration of TCN was lowered, the progression of cells out of S phase was more extensive (FIG. 5). Thus, it appeared that 0.1 μM, the lowest concentration of TCN studied, was sufficient to block entry of G_1 cells into S phase, but that the slowing of S-phase

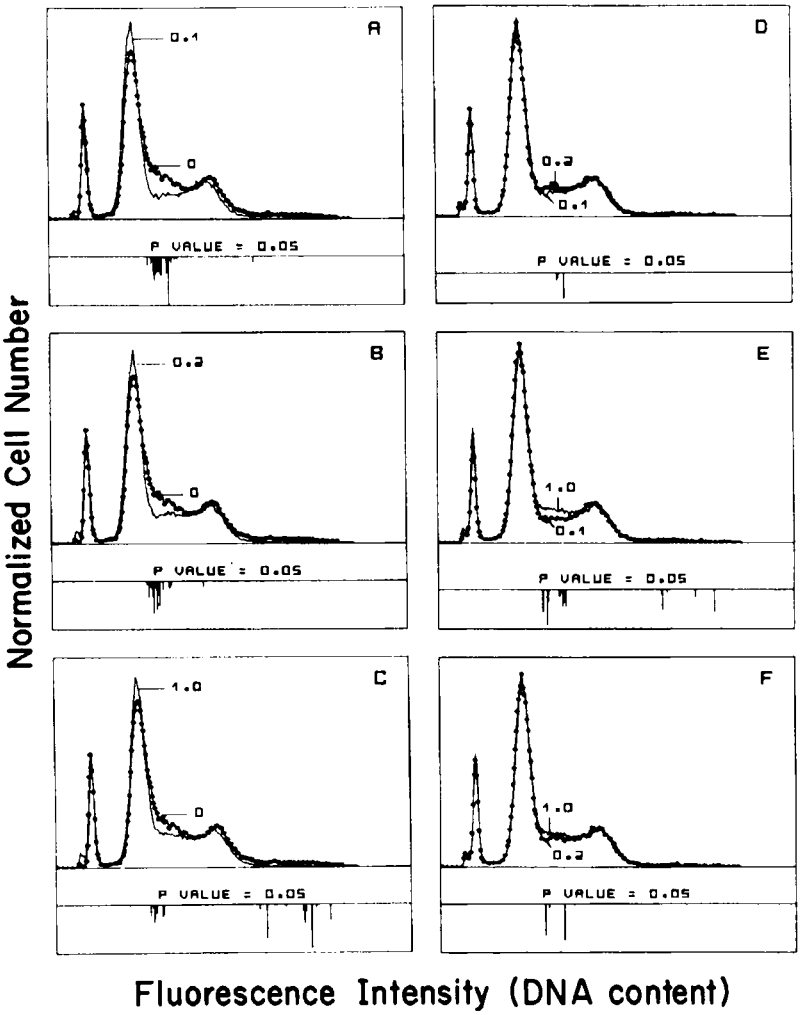


FIG. 5. Effect of various concentrations of TCN on the population distribution of DNA contents. L1210 cells were treated for 10 hr with TCN. Triplicate samples were prepared for flow cytometry by the propidium iodide technique as described previously,[7] and analysed using a Coulter Epics V cell sorter system. The peak with the lowest DNA content represents murine spleen cells added as a marker for DNA content. A-C, the averaged histograms from TCN-treated cells compared with the average control histogram (0) obtained from exponentially growing cells not treated with TCN; , control in A-C; , 0.1 μM TCN in A, 0.2 μM TCN in B, 1 μM TCN in C. D-F, paired comparisons of the averaged histograms from TCN-treated cells; , 0.2 μM TCN in D and F, 0.1 μM TCN in E; , 0.1 μM TCN in D, 1 μM TCN in E and F. In A-F, the bars shown in the P value portion of the chart indicate points of significant difference ($P < 0.05$) in a channel-by-channel t test of the pair of average histograms. (PARA 1 and NONPARA Programs, E.A.S.Y Computer System; Epics Divison, Coulter Corp., Hialeah, FL, U.S.A.) Thus clusters of bars indicate regions where the two cell cycle distributions being compared are significantly different.

progression became more pronounced as the concentration was increased from 0.1 μM to 1 μM. Therefore, it seems likely that TCN may inhibit DNA synthesis by (at least) two mechanisms, one that prevents the initiation of DNA synthesis and one that slows the completion of DNA synthesis.

One possible mechanism by which TCN might inhibit DNA synthesis would be purine nucleotide precursor depletion as a result of the inhibition of purine de novo synthesis by TCN shown by other investigators.[4,5,8] However, this inhibition did not appear to be the primary mechanism of action of TCN, since the addition of an exogenous purine source such as hypoxanthine to cell cultures simultaneously with TCN did not prevent cell growth inhibition or lethality.[4,5,6] Thus, if inhibition of DNA synthesis by TCN could be prevented by the addition of hypoxanthine, we would conclude that the inhibition of DNA synthesis was not the primary cause of cell death. To the contrary, hypoxanthine did not influence the inhibition of DNA synthesis by TCN (FIG. 6). Therefore, a role of DNA synthesis inhibition in the mechanism of TCN-induced lethality cannot be ruled out. Also, the inhibition of DNA synthesis was not caused by purine nucleotide depletion.

Accordingly, we considered it important to further investigate the mechanism of this inhibition. The first approach was to treat L1210 cells with 1 μM TCN for 1 hr, remove the TCN and then monitor the recovery of the rate of DNA synthesis (FIG. 7). Within 1 hr after removal of the TCN, the rate of DNA synthesis partially recovered. However, there was no further change for the duration of the 7-hr experiment, even though these cells were 100% viable, as shown in FIG. 2. Clearly, the cells were able to resume DNA synthesis, but not within 7 hr.

One possible explanation for the slowness of the recovery of DNA synthesis after TCN treatment could have been that TCN caused slowly repairable damage to the DNA. This hypothesis was tested using alkaline elution of cellular DNA.[9] The rate of elution of DNA from L1210 cells treated for 12 hr with 1 or 10 μM TCN was indistinguishable from that of control, untreated cells (FIG. 8). This result showed that TCN did not induce strand breakage in the DNA. The possibility of DNA-DNA or protein-DNA cross linking was also

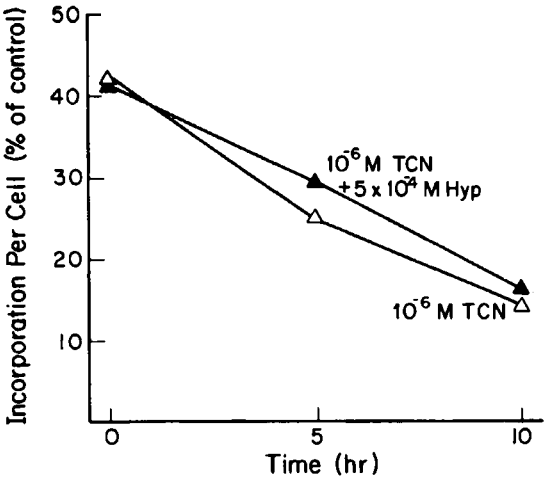


FIG. 6. Effect of TCN and hypoxanthine on ^{3}H-dThd incorporation. L1210 cells were treated continously with 1 μM TCN, alone (—△—) or in combination with 500 μM hypoxanthine (—▲—), for the indicated times. Then ^{3}H-dThd (1 μCi/ml) was added to the culture and the incorporation into acid-insoluble material was determined every 15 min for 1 hr.[7] The rate of incorporation per cell was calculated by a linear least squares fit of this data and expressed as the percentage of the rate for the time-matched control. The values are the averages of triplicate determinations in a single experiment.

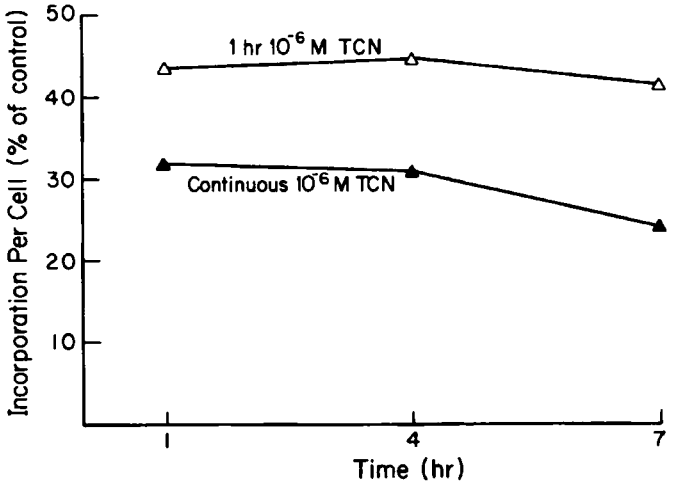


FIG. 7. Reversibility of inhibition of DNA synthesis by TCN. L1210 cells were incubated with 1 μM TCN for 1 hr, and then split into two groups. For the first group (—△—) the cells were removed from the TCN-containing medium at 0-time and placed in fresh, drug-free medium. For the second group (—▲—) the cells remained in the TCN-containing medium. At the indicated times ^{3}H-dThd (1 μCi/ml) was added to the culture and the rate of incorporation determined as described for FIG. 6. The values are the averages from two independent experiments, each having triplicate determinations.

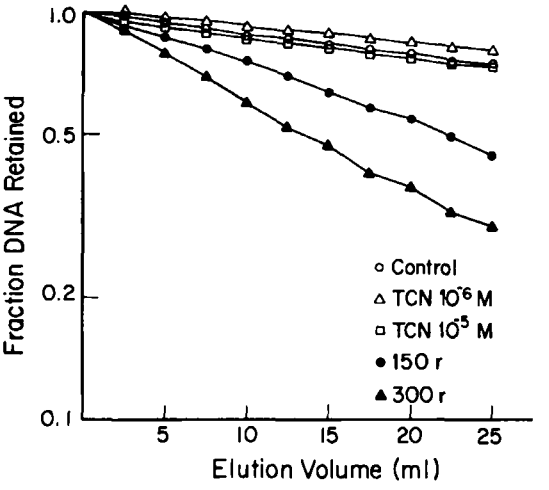


FIG. 8. Effect of TCN treatment and irradiation of cells on alkaline elution of DNA. L1210 cells were radiolabeled with 0.02 μCi/ml 2-^{14}C-dThd for 24 hr and exposed to TCN for 12 hr (during 13-24 hr of 2-^{14}C-dThd exposure): ○,0 M (control) △, 1 μM; □, 10 μM TCN. For a positive control, cells were radiolabeled with methyl-^{3}H-dThd (0.1 μCi/ml) for 20.5 hr and exposed to 150 (●) or 300 (▲) rads of ^{60}Co gamma rays. The cells from each treatment were suspended in Puck's saline G at 0°C and flowed by gravity onto separate filters. Cells were lysed and DNA was eluted with 30 ml elution solution (pH 12.1) at a flow rate of 0.05 ml/min. Fractions were collected every 60 minutes. The DNA remaining on the filter was hydrolyzed, and the radioactive material in this hydrolysate and in the eluted fractions was determined by liquid scintillation counting. The fraction of the total radioactivity (sum of eluted fractions + filter hydrolysate) that remained on the filter was then plotted for each fraction. An increased rate of elution in comparison to that of the untreated control indicated the presence of single strand breaks in the DNA.[9] Each point represents the mean of 2-3 determinations from separate experiments.

examined by alkaline elution of DNA from TCN-treated and control cells which were irradiated with 300 rads of ^{60}Co γ-rays, immediately before elution. Under these conditions, either type of cross linking would cause retention of the DNA from drug-treated cells in comparison with control cells.[9] No effect was detectable (data not shown), indicating that TCN did not cause cross linking of either type. Another possible explanation for the slowness of recovery of DNA synthesis would be a retention of TCN-P within the cells.

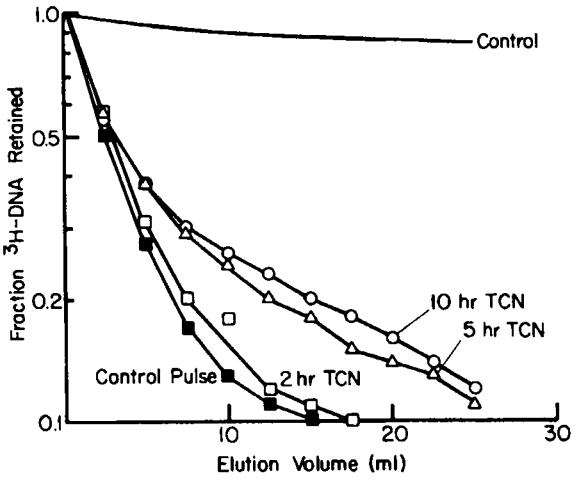
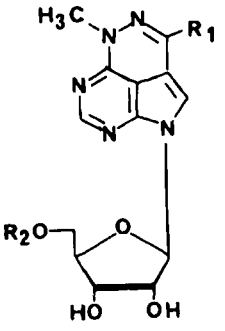


FIG. 9. Effect of pretreatment with TCN on the rate of elution of pulse-labeled DNA. L1210 cells were incubated for 0 (■), 2 (□), 5 (△), or 10 (○) hr with 0.1 μM TCN. Then they were pulse-labled with ^{3}H-dThd (15 min, 5 μCi/ml, 1 μM dThd) and their DNA characterized by alkaline elution as described for FIG. 8. However, in this experiment the control pulse-labeled DNA eluted rapidly because the ^{3}H-dThd was newly incorporated and had not been elongated and/or ligated into high molecular weight species. A typical high molecular weight control (—) is shown for comparison (approximately 24 hr, 0.1 μCi/ml ^{3}H-dThd) The values shown are the averages of 2-3 determinations in separate experiments.

Recently, we have initiated experiments designed to test the hypothesis that TCN may selectively inhibit the initiation of DNA synthesis. One strategy was to characterize the average length of actively elongating DNA chains. If chain initiation were selectively inhibited, while elongation proceeded, this average length would be expected to increase. This question was approached experimentally by preincubating L1210 cells with 0.1 μM TCN for various times, then pulse labeling with ^{3}H-dThd (15 min, 5 μCi/ml, 1 μM dThd) and characterizing the relative molecular weight of the ^{3}H-labeled products by alkaline elution (FIG. 9). After 5 hr or more of preincubation with TCN, the rate of elution of ^{3}H-DNA was significantly slowed, indicating that the actively elongating DNA chains were longer on the average than in controls. Thus, under these conditions it appeared that TCN might preferentially inhibit



	R_1	R_2
4	$-NH_2$	$-H$
5	$-N=CH-N(CH_3)_2$	$-H$
6	$-NH_2$	$-P(=O)(OH)-OH$

SCHEME 2

initiation of new DNA chains, as opposed to elongation of existing DNA chains. Further experiments are in progress to confirm this conclusion and to elucidate the mechanisms involved.

The development of TCN for clinical application required a more water-soluble derivative since TCN was soluble only at or below pH 2.0. First, the 6-dimethylaminomethyleneamino derivative 5 was prepared by treatment of TCN (4) with dimethyl formamide dimethylacetal.[10] Second, the 5'-monophosphate derivative 6 was prepared by treatment of TCN (4) with triethylphosphate and phosphorus oxychloride at 0°.[10,11] Both of these compounds were much more water soluble than TCN. Initial evaluation of cytotoxicity *in vitro* showed that while 5 was about 20 times less effective than TCN (4) in inhibiting L1210 cell growth, TCN-P(6) retained the full cytotoxic potency of the parent compound (FIG. 10).[12] This data proved to be

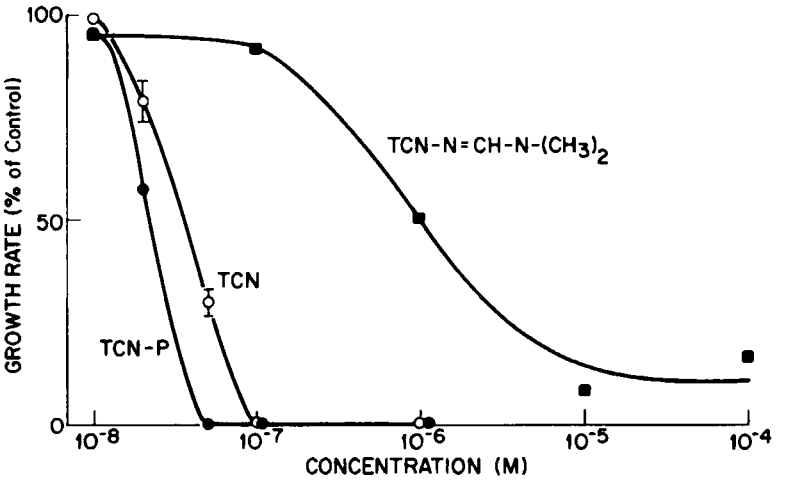
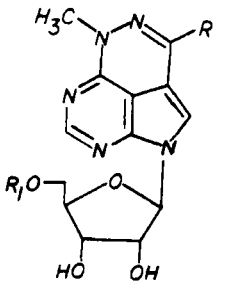


FIG. 10 Effect of TCN and its water-soluble derivatives on the growth rate of L1210 cells. The growth rates were determined from plots of log of cell number against time over a three-day continuous treatment with the indicated concentration of drug, as described previously.[12] O, TCN (4); ■, 5; ●, 6.

predictive of the in vivo antitumor response (TABLE 1). The dimethylaminomethyleneamino derivative 5 was essentially inactive in prolonging the lifespan of L1210-bearing mice. On the other hand, TCN-P proved to be a fully active, water soluble derivative that was then studied further as a possible clinical candidate. Both TCN and TCN-P were also quite effective against certain solid tumors, such as the MX-1 human breast carcinoma xenograft in nude mice and the CD8F murine mammary adenocarcinoma.[13]

TCN-P was then entered into Phase I clinical trials. While these were in progress, a preclinical study was undertaken by Cobb and coworkers[14] to obtain a preliminary indication of which human tumors might be most effectively treated with TCN-P. In the subrenal capsule assay, fragments of tumor biopsy specimens were implanted in the subrenal capsules of normal mice and the change in their size determined after 6 days. In this assay TCN-P compared favorably with several established antitumor drugs in inhibiting growth of human tumor fragments. Activity was particularly remarkable against cervical and ovarian carcinomas, with 7 tumors responding out of 8 tested for cervical, and 7 out of 10 for ovarian.

TABLE 1



-R	$-R_1$	Experiment 1 Dose per Injection (mg/kg)	Experiment 1 ILS (% of control)	Experiment 2 Dose per Injection (mg/kg)	Experiment 2 ILS (% of control)
$-NH_2$	-H	64	*	50	-5
		32	*	37.5	*
		16	38	25	17
		8	26	18.7	37
		4	60	12.5	48
				9.4	26
				6.25	29
$-N=CH-N(CH_3)_2$	-H	900	26		
		600	26		
		400	15		
		200	4		
		100	8		
$-NH_2$	$-\overset{O}{\overset{\|}{P}}-OH$ $\overset{\|}{OH}$			62.5	43
				46.9	74
				31.2	59
				23.4	53
				15.6	25
				11.7	32
				7.81	32

*toxic test

In vivo Antitumor Evaluation of 4 and its Water Soluble Derivatives Against L-1210 Mouse Leukemia.[13] The dose listed was administered daily for nine days, starting on the day after inoculation of the animals with tumor cells. The increase in life span (ILS) in comparison to untreated control animals is presented for the two experiments where the respective water soluble derivatives were compared directly with the parent nucleoside.

In the Phase I studies several partial responses were noted in the trial utilizing a 5-day continuous infusion schedule,[15] and the primary clinical toxicities were hyperglycemia and hepatotoxicity.[15,16] The hyperglycemia was manageable with insulin administration, but the hepatotoxicity was dose-limiting. Gastrointestinal toxicity was infrequent and myelosuppression was not observed, suggesting that TCN-P may be useful in chemotherapeutic combinations with other drugs with non-overlapping toxicities, which would include the majority of anticancer drugs. TCN-P is currently in Phase II trials to further investigate its clinical antitumor efficacy.

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