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Inhibition of Transfer Ribonucleic Acid Methylase Activity from Several Human Tumors by Nicotinamide and Nicotinamide Analogs*

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ABSTRACT: Previous studies (Halpern, R. M., Chaney, S. Q., Halpern, B. C., and Smith, R. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 602) have demonstrated that a low molecular weight inhibitor of tRNA methylase exists in normal rat liver. The isolation of this substance and subsequent structure elucidation has revealed it to be nicotinamide. In these present studies, we have demonstrated that nicotinamide is capable of inhibiting tRNA methylase activity obtained from a human tumor cell line grown in tissue culture (KB cells). Furthermore, several structural analogs of nicotinamide, for example, thionicotinamide, 6-aminonicotinamide, and pyridine-3-carboxaldehyde, were also capable of inhibiting the KB cell enzyme. Finally, we have demonstrated that nicotinamide is able to inhibit tRNA methylase activity in extracts prepared from several human malignancies: three adenocarcinomas of the bowel, a reticulum cell sarcoma, and a seminoma. Nicotinamide is without effect on the tRNA methylase activity prepared from normal tissues removed from the areas surrounding the tumors.

The greater tRNA methylase activity in extracts prepared from malignant cells (Berquist and Mathews, 1962; Tsutsui *et al.*, 1966; Chaney *et al.*, 1970) as well as from cells isolated from fetal tissue (Simon *et al.*, 1967; Hancock, 1967; Kerr, 1970) has been attributed to the absence of inhibitors of that enzyme in those cells. In 1970, Kerr identified a nondialyzable heat-labile, trypsin-sensitive protein from adult normal tissue and Novikoff tumor, which was capable of inhibiting tRNA methylation. Recently we have shown that a dialyzable inhibitor (Chaney *et al.*, 1970) is present in normal adult tissue and absent in the malignant tissues which we have examined. We have purified the dialyzable inhibitor and subsequent analysis has revealed it to be nicotinamide (Halpern *et al.*, 1971). Kerr has recently reported the isolation of two substances from normal adult tissue which "in concert" are capable of causing inhibition of tRNA methylase. One of these is a low molecular weight fraction (mol wt <700) which by itself is inactive, but when added to the second high molecular weight substance has increased inhibitory activity. The high molecular weight component is apparently absent from embryonic and tumor tissues (Kerr, 1971).

These present studies were undertaken to determine whether or not analogs of nicotinamide possessed inhibitory activity against tRNA methylase isolated from adult rat liver, a W-256 tumor, and W-256 tumor cells grown in tissue culture.[1] In addition, we wished to examine whether the tRNA methylase prepared from human malignant cells was capable of being inhibited by nicotinamide; therefore, we prepared tRNA methylase from KB cells grown in culture and from several human malignancies: three adenocarcinomas of the bowel, a reticulum cell sarcoma, and a seminoma. All were strikingly inhibited by nicotinamide. Kinetic evaluation of the effects of nicotinamide as well as some of the more active analogs against KB cell and W-256 cell tRNA methylase are also presented.

Materials and Methods

Preparation of tRNA Methylase. KB CELLS. KB cells were grown using a roller cell apparatus to a density of 5×10^6/ml. Cells were harvested and washed by suspension in isotonic sterile saline. They were then resuspended at a density of 0.01 g/ml in Tris–magnesium chloride buffer (Tris, 0.01 M, pH 8–magnesium chloride 0.01 M). The cells were broken by homogenization at 4° in a Sorvall OmniMixer run at top speed for approximately 1 min. After centrifugation at 4° for 10 min at 10,000*g*, the supernatant was removed and centrifuged at 105,000*g* for 60 min at 4°. The 105,000*g* supernatant was removed and used as a cell-free tRNA methylase preparation.

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[1] Walker-256.

Rat Liver and W-256 Tumor. The tumors used were subcutaneously implanted W-256 neoplasms. Livers and tumors were rapidly removed from exanguinated ether-anesthetized Sprague-Dawley rats and immediately immersed in a cold buffer (0°) containing 0.25 M sucrose, 0.01 M magnesium chloride, and 0.01 M Tris, pH 8 (SMT buffer). The tissues were minced in two volumes of cold SMT buffer and then broken. The liver was disrupted in a Teflon-glass homogenizer while the tumors were disrupted at high speed for 1 min in a Virtis homogenizer. The homogenates were centrifuged at 4° for 10 min at 17,300*g*, the surface lipids were removed by aspiration, and the resulting supernatant solution was centrifuged at 2° for 60 min at 105,000*g*. The remaining surface lipid layer was removed by aspiration. This 105,000*g* delipidized supernatant solution will be referred to as the crude enzyme preparation. The pH of the supernatant solution was reduced to 5 by the addition of 1 N acetic acid and was then centrifuged at 17,300*g* for 10 min at 4°. The pH 5 precipitate was then suspended in very minimal amounts of a buffer containing 0.01 M Tris (pH 8.2), 0.002 M EDTA, and 0.005 M dithiothreitol to form a thick paste. This paste was then dialyzed for 4 hr *vs.* SMT buffer and then centrifuged at 17,300*g* for 10 min at 4°. The supernatant solution was then lyophilized in five-drop aliquots and stored dry at −10°. For assay, the dried enzyme preparation was redissolved in 5 drops of water and was unstable if refrigerated or frozen again. For the following studies, a dialyzed and lyophilized 105,000*g* supernatant solution (crude enzyme) and a dialyzed and lyophilized pH 5 precipitate prepared from the 105,000*g* supernatant solution of both rat liver and W-256 tumor were used.

Both of these preparations contained tRNA methylase activity and the enzyme preparation used in each test is specified in each table.

Human Tumors. Human cancers removed at the time of surgery, as well as normal surrounding tissue, were placed in sterile saline and immediately chilled at 4°. Crude enzymes were prepared from both the neoplasms and the normal tissue within 24 hr by the above described procedure.

Enzyme Assay. Rat liver, W-256 tumor enzyme, and KB cell enzymes were assayed with or without the addition of inhibitors according to a previously described method (Halpern *et al.*, 1971; Kerr, 1970). Aliquots (100 μl) were removed in duplicate from each reaction mixture and applied to 2.5 cm Whatman No. 3MM disks and hot air-dried. Each duplicate set of disks was placed in a beaker and covered with 10% trichloroacetic acid for 30 min, followed by 5% trichloroacetic acid for 15 min. One set was heated at 90° for 30 min in 5% trichloroacetic acid while the other remained in cold 5% trichloroacetic acid. Both sets were then allowed to stand in cold 5% trichloroacetic acid for 15 min, ethanol–ether (1:1, v/v) at 37° for 15 min, and finally ether at room temperature for 15 min. The disks were then dried and counted in 10 ml of toluene scintillation solution in a Beckman LS-100 liquid scintillation counter.

The counts remaining on the filter after the hot trichloroacetic acid wash represented the incorporation of methyl groups into protein, since hot trichloroacetic acid renders methylated tRNA soluble. The determination of the amount of methyl groups incorporated into tRNA was calculated by subtracting the total counts incorporated into protein (hot trichloroacetic acid insoluble cpm) from the total counts incorporated into both tRNA and protein (cold trichloroacetic acid insoluble counts per minute). A second method of measuring the counts per minute incorporated into tRNA involved assaying duplicate sets of incubations, one in the presence of *Escherichia coli* B tRNA and the other in the absence of tRNA. *E. coli* K_{12} tRNA could be used with the same results. Subtraction of the counts incorporated in the absence of tRNA (protein, endogenous tRNA) from the total counts incorporated in the presence of tRNA represents incorporation of methyl groups into tRNA alone. Experiments with the KB cell extract (105,000*g* supernatant) indicate that either of the above methods are sufficient to correct for incorporation of methyl groups into protein and at least in the case of the KB cell extract give similar results. Depending on the level of enzyme utilized, a 5- to 10-fold increase in counts per minute incorporated into trichloroacetic acid insoluble material is observed in the presence of *E. coli* B tRNA. We have shown that most of this increase (>95%) is due to incorporation into tRNA (hot trichloroacetic acid soluble) and not protein.

All of the K_m values and K_i values were obtained mathematically using the formula derived by a linear regression analysis of the data. The basic incubation mixture contained the following substances (amounts in micromoles) in a final volume of 0.20 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 8; *E. coli* B tRNA, 100 μg; KB S-100 extract containing 100–400 μg of protein; and inhibitor at the concentrations indicated. Reactions were incubated at 37° for 30–60 minutes. The velocity in all cases is expressed as pmoles of methyl group incorporated into tRNA per 30 min.

Nicotinamide, as well as all of the substituted pyridine compounds used in this study, were obtained from Aldrich Chemical Co. These compounds were checked for purity by melting point and thin-layer chromatography (tlc). If more than one component was present, they were purified by recrystallization or distillation until a single component was shown to be present on tlc or until the melting point was in agreement with published literature values. Nicotinamide used for the studies performed on rat liver, W-256, and human tumor methylases was obtained from Sigma Chemical Co. *S*-Adenosyl-L-methionine-*methyl*-^{14}C was obtained from International Chemical and Nuclear Corp. and had a specific activity of 40–55 mCi/mmole.

Results and Discussion

KB Cell tRNA Methylase. Crude 105,000*g* supernatant prepared from KB cells was found to contain considerable tRNA methylase activity as previously described (Zeleznick, 1967). When tRNA methylase activity was measured at varying protein concentrations, the results shown in Figure 1 were obtained. As can be seen, the incorporation of methyl-^{14}C groups into *E. coli* B tRNA was nearly linear over the entire range of protein concentrations. This was in contrast to the results obtained with crude 105,000*g* supernatant prepared from adult animal tissues; *e.g.*, rat liver, rabbit brain, rabbit liver (Kerr, 1970), or pig brain (Swiatek *et al.*, 1971), but similar to the results obtained with the dialyzed pH 5 preparation of adult rat liver and W-256 tumor extracts (Halpern *et al.*, 1971). Incorporation was linear with time over a 60-min incubation period.

On the basis of the above results, it appears as if KB cell extracts either lack, or contain very small quantities of, the natural tRNA methylase inhibitor(s). In that regard, these tissue culture cells derived from a human tumor cell line more nearly resemble the other tumor cell lines examined by us (Halpern *et al.*, 1971). Because KB cells represent a human

TABLE I: Inhibition of KB tRNA Methylase by Nicotinamide Analogs.[a]

Compound	Concn (M)	% I
Nicotinamide	5×10^{-4}	53
Nicotinic acid hydrazide	5×10^{-4}	0
3-Pyridine aldoxime	5×10^{-4}	0
Thionicotinamide	5×10^{-4}	37
Nicotinamide *N*-oxide	5×10^{-4}	0
6-Aminonicotinamide	5×10^{-4}	22
6-Chloronicotinamide	5×10^{-4}	0
Isonicotinamide	5×10^{-4}	0
Methyl nicotinate	5×10^{-4}	0
Pyrazinamide	5×10^{-4}	0
3-Cyanopyridine	5×10^{-4}	0
N-Methylnicotinamide	5×10^{-4}	0
3-Hydroxypicolinamide	5×10^{-4}	0
3-Pyridinecarboxaldehyde	5×10^{-3}	22
4-Pyridinecarboxaldehyde	5×10^{-3}	15
2-Pyridinecarboxaldehyde	5×10^{-3}	14
3-Acetylpyridine	5×10^{-3}	11
Hexahydronicotinamide	5×10^{-3}	8
3-Methylpyridine	5×10^{-3}	27
Indole-3-acetic acid hydrazide	5×10^{-3}	6
Nicotinic acid piperidinamide	5×10^{-3}	2
3-Pyridylcarbinol	5×10^{-3}	15

[a] Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris Buffer, 7.4, pH 8.5; *E. coli* B tRNA, 100 μg; KB S-100 extract; 100–400 μg of protein, and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

tumor cell line grown in tissue culture, it was of interest to examine the effect of nicotinamide on the tRNA methylase prepared from those cells. In addition, since the crude 105,000*g* supernatant was shown (Figure 1) to contain either a very low amount of, or to lack completely, the natural inhibitor(s) of tRNA methylase previously reported (Halpern *et al.*, 1971; Kerr, 1971), it was ideally suited for testing the effects of inhibitor(s) of this reaction without resorting to the extensive purification procedures needed for preparation of an inhibitor-free enzyme.

Preliminary experiments (Table I) revealed that nicotinamide was indeed capable of inhibiting tRNA methylation. As noted, none of the compounds tested seemed to possess greater activity against the KB cell tRNA methylase than nicotinamide itself, although several of the compounds did appear to possess significant inhibitory activity; namely, thionicotinamide and 6-aminonicotinamide were found to have 37 and 22% inhibition at the same concentration of nicotinamide which gave 53% inhibition. At ten times higher concentrations, pyridine-3-, pyridine-4-, and pyridine-2-carboxaldehyde were seen to exhibit 22, 15, and 14% inhibition, respectively, of KB cell tRNA methylase. In addition, 3-methylpyridine at 5×10^{-3} M was also found to inhibit the reaction to an extent of 27%. All these compounds exhibited virtually no inhibition at 5×10^{-4} M. The inhibitory activity of the two most active compounds, thionicotinamide and 6-

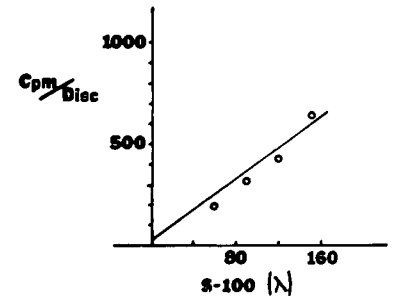

FIGURE 1: *In vitro* methylation of *E. coli* B tRNA by S-100 from KB cells. Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 7.4; *E. coli* B tRNA, 100 μg; KB S-100 extract containing 100–400 μg of protein; and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

aminonicotinamide, as well as nicotinamide itself, was next analyzed by a double-reciprocal plot of $1/v$ *vs.* $1/(S)$ in order to determine the type and the extent of inhibition. Substrate concentration was varied over a wide range in the presence and absence of nicotinamide, and the velocity was measured. The K_m for *S*-adenosylmethionine was calculated (Figure 2) and found to be 3.5×10^{-6} M. This compares to a value of 1.6×10^{-6} M obtained for the enzyme isolated from W-256 tumor (Halpern *et al.*, 1971). Examination of the data in Figure 2 indicates that nicotinamide exhibits a mixed type of inhibition. Using the mathematical treatment given for a competitive inhibitor, a K_i value of 1.25×10^{-3} M was calculated for nicotinamide.

In order to substantiate the type of inhibition as well as compare the inhibitory constant (K_i value), a second experiment was performed in which inhibitor concentration was varied at two different concentrations of *S*-adenosylmethionine. A Dixon plot (Dixon and Webb, 1964) (Figure 3), in which inhibitor concentration was plotted against the reciprocal velocity, also indicated that the type of inhibition observed was mixed and the K_i value for nicotinamide was calculated as 2×10^{-3} M; this was in good agreement with the Lineweaver–Burk method.

Experiments were next initiated in which the type and ex-

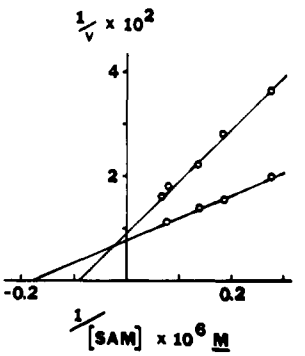

FIGURE 2: Lineweaver–Burk plot. *In vitro* methylation of *E. coli* B tRNA by KB cell S-100, inhibition by nicotinamide. Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 7.4; *E. coli* B tRNA, 100 μg; KB S-100 extract containing 100–400 μg of protein; and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

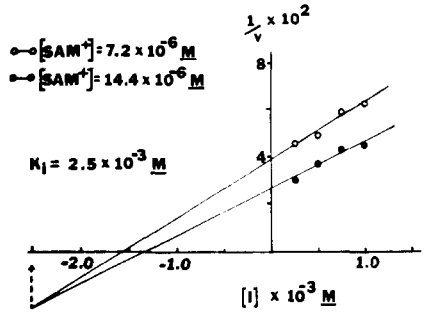

FIGURE 3: Dixon plot. *In vitro* methylation of *E. coli B* tRNA by KB cell S-100, inhibition by nicotinamide. Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 7.4; *E. coli* B tRNA, 100 μg; KB S-100 extract containing 100–400 μg of protein, and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

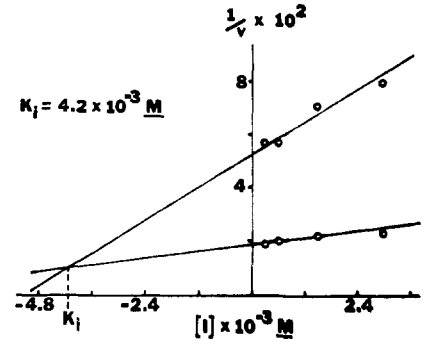

FIGURE 5: Inhibition of KB tRNA methylase, by thionicotinamide. Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 7.4; *E. coli* B tRNA, 100 μg; *S*-adenosyl-L-methionine-^{14}C, 1.9 $\times$ 10^{-6} M; *S*-adenosyl-L-methionine-^{14}C, 7.6 $\times$ 10^{-6} M; KB S-100 Extract containing 100–400 μg of protein; and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

tent of inhibition exhibited by two of the more potent analogs were examined. As can be seen from Figure 4, thionicotinamide also appeared to exhibit mixed kinetics, but the straight lines obtained in the double-reciprocal plots appeared to intercept more closely to the ordinate ($1/v$) than in the case of nicotinamide. It thus appears that the type of inhibition observed with thionicotinamide more nearly approximated competitive kinetics. For purposes of calculating a K_i value, a second experiment was run in which inhibitor concentration was varied at two different substrate concentrations. When these data were analyzed by the method of Dixon (Dixon and Webb, 1964), the two lines intercepted very close to the abcissa, thus indicating that the type of inhibition, although mixed, more nearly approximated the competitive type. A K_i value was calculated from this data (Figure 5) and a value of 4.2 $\times$ 10^{-3} M was obtained.

Similar studies were performed with 6-aminonicotinamide and using the method of Dixon (Dixon and Webb, 1964), this compound was found to exhibit nearly pure competitive kinetics and a K_i value of 3.7 $\times$ 10^{-3} M was obtained (Figure 6).

W-256, Rat Liver, and Human Seminoma Tumor tRNA Methylase. Similar kinetic studies on rat liver and W-256 methylases were performed using only nicotinamide and these were published earlier (Halper, 1971). Basically the same results were obtained as with the KB cell line.

Very similar results were also obtained when a variety of analogs were tested on rat liver, W-256, and a human seminoma tumor methylase (Table II). Thionicotinamide (at a concentration of 0.4 mM) exhibited 38 and 28% inhibition on W-256 and rat liver methylases, respectively, but no other analog inhibited the enzymes to the degree that nicotinamide did.

Finally, tRNA methylase was prepared from several human adenocarcinomas of the colon and a human carcinoma of the kidney. The type of enzyme preparation used is indicated in Table III. Crude extracts were prepared from the carcinomas and from the surrounding normal tissues as well. The results presented in Table III indicate that nicotinamide at 5 mM concentration was capable of causing from 41–57%

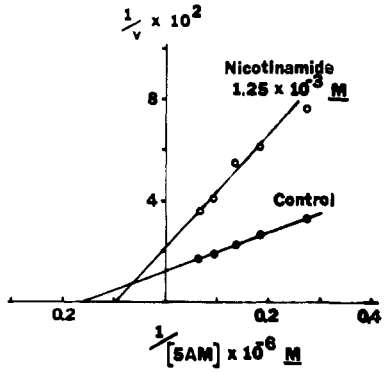

FIGURE 4: Inhibition of KB tRNA methylase by thionicotinamide. Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 7.40 *E. coli* B tRNA, 100 μg; thionicotinamide, 2 $\times$ 10^{-3} M, final concentration; KB S-100 extract containing 100–400 μg of protein; and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

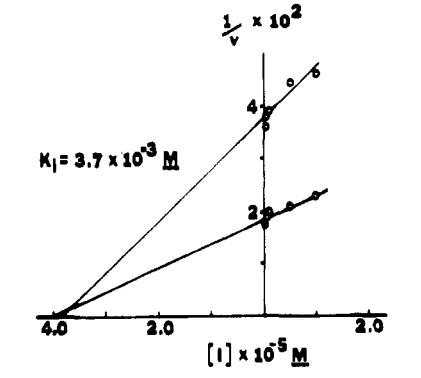

FIGURE 6: Inhibition of KB tRNA methylase, by 6-aminonicotinamide. Reactions contained the following (in micromoles) in a final volume of 0.2 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0008; magnesium acetate, 1.84; mercaptoethanol, 5.2; Tris buffer, pH 8.5, 7.4 ; *E. coli* B tRNA, 100 μg; *S*-adenosyl-L-methionine-^{14}C 1.9 $\times$ 10^{-6} M; *S*-adenosyl-L-methionine-^{14}C, 7.6 $\times$ 10^{-6} M; KB S-100 extract containing 100–400 μg of protein, and inhibitor at the concentrations indicated. Reactions were incubated at 37°, for 30–60 min.

TABLE II: Effect of Analogs on tRNA Methylase from Rat Liver, Walker-256, and Human Seminoma.[a]

	Concn (mM)	W-256	% Inhibition Methylase from Rat Liver	Seminoma
Nicotinamide	5.0	43	66	10
Nicotinic acid hydrazide	5.0		0	0
3-Pyridinecarboxaldehyde	4.0		22	5
Thionicotinamide	0.4	38	28	
Nicotinamide *N*-oxide	5.0		11	0
6-Aminonicotinamide	5.0	16	12	
6-Chloronicotinamide	5.0	0	0	0
Isonicotinamide	5.0	19	36	
α-Picolinamide	5.0	16	30	24
3-Acetylpyridine	5.0		13	24
N-Methylnicotinamide	5.0	0	0	16
3-Cyanopyridine	5.25		24	0

[a] Reactions contained the following (in micromoles) in a final volume of 0.4 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 40 mCi/mmole), 0.0056; dithiothreitol, 0.16; Tris Buffer, pH 9, 25; *E. coli* K-12 submethylated tRNA, 50 μg and enzyme in SMT Buffer (0.25 M sucrose, 0.01 M $MgCl_2$, and 0.01 M Tris, pH 8) as indicated in the following: W-256, 160 μg of pH 5.0 precipitated protein; rat liver, 190 μg of pH 5.0 precipitated protein; Seminoma 230 μg of crude 100,000*g* supernatant.

TABLE III: Effect of Nicotinamide (5 mM) on tRNA Methylases Obtained from Human Neoplasms.[a]

Tissue	% Inhibn	Type of Prepn
Colon		
Adenocarcinoma	41	Crude
Normal	0	Crude
Colon		
Adenocarcinoma	57	Crude
Normal	5	Crude
Colon		
Adenocarcinoma	50	Crude
Normal	0	Crude
Colon		
Adenocarcinoma	38	pH 5.0
Normal	0	pH 5.0
Kidney		
Carcinoma	54	Crude
Normal	0	Crude
Testes		
Seminoma	20	Crude
Normal	23	Crude

[a] Reactions contained the following (in micromoles) in a final volume of 0.4 ml: *S*-adenosyl-L-methionine-^{14}C (specific activity 55 mCi/mmole), 0.0056; dithiothreitol, 0.16; Tris Buffer, 25, pH 9; *E. coli* K-12 submethylated tRNA, 50 μg; and enzyme in SMT Buffer (0.25 M sucrose, 0.01 M $MgCl_2$, and 0.01 M Tris, pH 8), 190–500 μg.

inhibition of the tumor cell enzymes, but produced little or no inhibition of the tRNA methylases prepared from the surrounding normal tissues. The same results were obtained whether the 105,000*g* supernatant or the pH 5 precipitate was used as the source of enzyme (Table III).

In summary, our initial observations on the isolation and identification of nicotinamide as a natural inhibitor of tRNA methylation from rat liver, which is capable of inhibiting W-256 tumor tRNA methylase as well as inhibitor-free tRNA methylase isolated from rat liver, has been extended to other systems. We have shown that nicotinamide will inhibit the tRNA methylases prepared from KB cells grown in tissue culture and that the K_i value is approximately equal to that previously obtained for the rat liver or W-256 tRNA methylase, although the type of inhibition for the KB cell enzyme appears to differ from that observed with either the W-256 or rat liver preparation. In addition, nicotinamide, as well as several of its analogs, notably thionicotinamide, 6-aminonicotinamide, and pyridine-3-carboxaldehyde, were capable of inhibiting the W-256 and rat liver enzymes and/or the KB cell methylase.

Finally, nicotinamide has been shown to inhibit the tRNA methylases prepared from four human tumors, but to be inactive against the tRNA methylase prepared from normal human tissues which surround the tumor. Further studies are in progress with regard to the effect of nicotinamide and its analogs on tumor growth in animals as well as on the effect of other pyridine derivatives on the cell-free enzymes described in this report.

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