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SHORT COMMUNICATIONS

Inhibition of nicotinate phosphoribosyltransferase in human platelet lysate by nicotinic acid analogs

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THE HUMAN platelet accumulates radioactivity by a saturable process when incubated with 7-[^{14}C] nicotinic acid. Such accumulation is due to incorporation of 7-[^{14}C]nicotinic acid into NAD, NADP, and their precursors which do not readily diffuse from the cell.[1] Accumulation and incorporation are hindered by nicotinic acid analogs, salicylic acid, and other anti-inflammatory drugs.[1,2] This study involves the structural requirements for inhibition of nicotinate phosphoribosyltransferase in human platelet lysate by nicotinic acid analogs.

Materials. The following reagents were purchased from commercial sources: 7-[^{14}C]nicotinic acid (sp. act., 59·1 mc/m-mole; Amersham/Searle); ATP, phosphorylribose-1-pyrophosphate, NAD, NADH, NADP, and nicotinamide (Mann Research Laboratory); salicylic acid (Matheson); pyridine (Mallinckrodt); nicotinamide mononucleotide (Sigma); and benzoic, isonicotinic, picolinic, 3-pyridylsulfonic, and 3-pyridylacetic acids (Aldrich). 3-Pyridylsulfonamide was synthesized by Dr. John Plati and the other nicotinic acid analogs by Dr. Alden Beaman of Hoffmann-La Roche. Nicotinic acid mononucleotide (NaMN) was prepared according to the method of Wagner.[3]

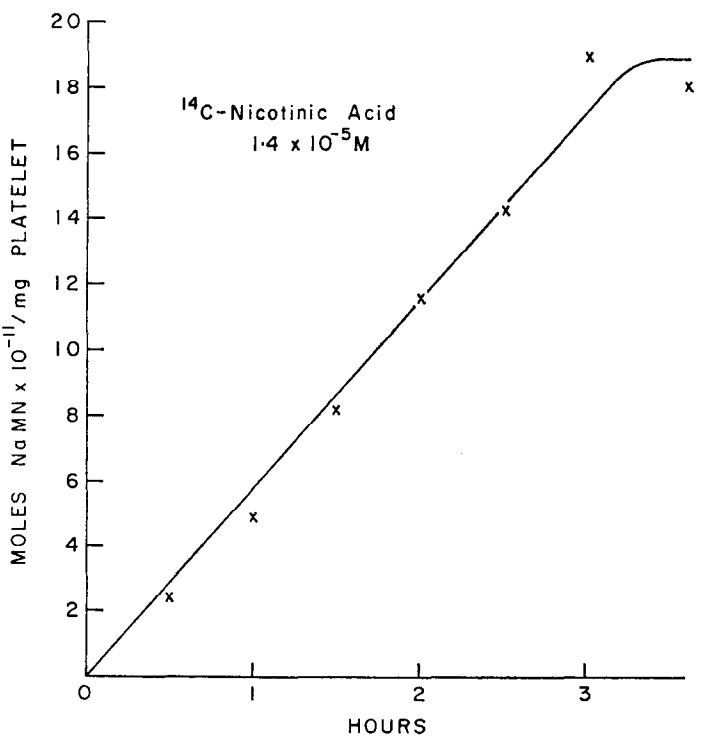

FIG. 1. Incorporation of 7-[^{14}C]nicotinic acid (14 μM) into NaMN by human platelet lysate with time. Velocity expressed as moles of NaMN formed $\times$ 10^{-11} per milligram of platelets. Results of a representative experiment are shown. See text for composition of incubation medium.

Methods. For each K_i determination, approximately 200 mg of human platelets, isolated as previously described,[1] were lysed in 10 vol. of distilled water. Aliquots of the lysate (0·2 ml) were incubated in Ca^{2+}-free, Krebs–Ringer bicarbonate buffer modified to contain: Mg^{2+}, 3·3 mM; the Mg^{2+} salt of phosphorylribose-1-pyrophosphate (PRPP), 1·5 mM; and various concentrations of 7-[^{14}C]nicotinic acid and its analogs. Such incubations were carried out in total volumes of 0·5 ml in a metabolic shaker under 95% O_2–5% CO_2 for 1 hr at 37°. The reaction was stopped by immersing the tubes in a solid CO_2–acetone mixture. Subsequently, "carrier" nicotinic acid and NaMN were added to the lysate and the compounds separated on thin layers of cellulose according to the method of Hagino *et al.*[4] The compounds were visualized by ultraviolet light, scraped from the plates, suspended in gelled scintillation medium, and the radioactivity was determined as previously described.[1] Velocity was expressed as moles of NaMN formed per milligram of platelets per hour. Parallel controls accompanied each K_i determination. Since the yield of platelets from human blood is small, purification of the enzyme was not possible.

When incubated with 7-[^{14}C]nicotinic acid and PRPP, human platelet lysate incorporates radioactivity into NaMN. Such incorporation was linear up to 3 hr at a nicotinic acid concentration of 14 μM (Fig. 1). Similar linearity was also observed at concentrations of 6·8, 17 and 34 μM. Figure 2

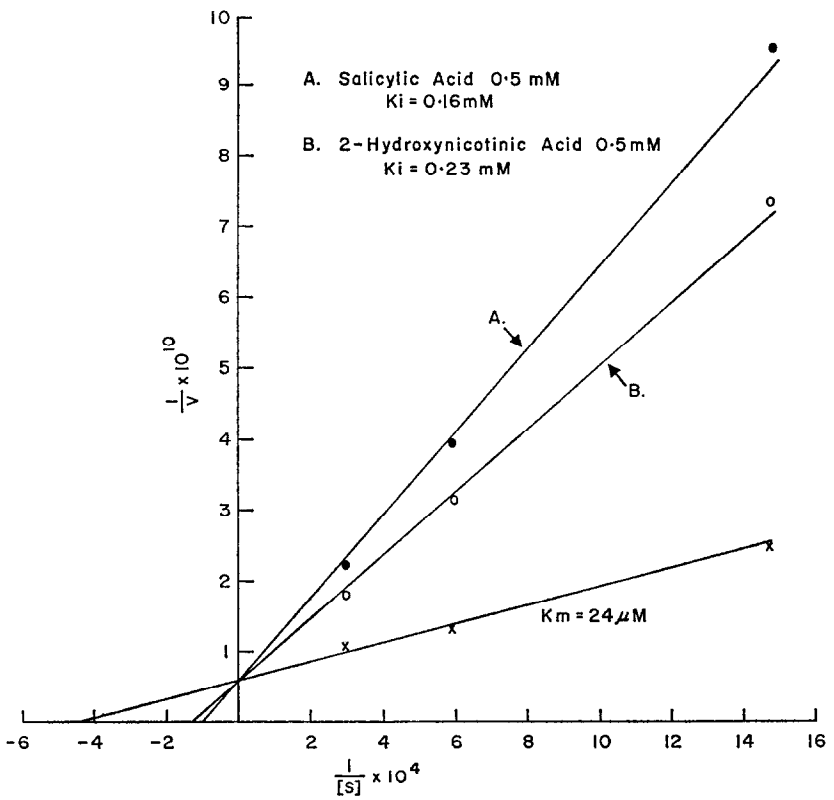

FIG. 2. Inhibition of nicotinate phosphoribosyltransferase in human platelet lysate by 2-hydroxynicotinic acid and salicylic acid. Velocity is expressed as moles of NaMN formed per milligram of platelets per hour; substrate concentration, moles per liter.

reveals the apparent K_m for nicotinic acid to be 24 μM where PRPP and Mg^{2+} were in excess. Incubation in Mg^{2+}-free buffer diminished the velocity approximately 50 per cent but left the apparent K_m unchanged. Although EDTA (3·3 mM) diminished the velocity by 50 per cent in the presence of Mg^{2+} (3·3 mM), no further reduction occurred with EDTA in the absence of $Mg.^{2+}$ Addition of NaF (1 mM) and ATP (0·5 mM) both separately and together did not change the K_m or velocity. NAD, NADP, and NADH also had no effect at 0·5 mM.

Of the several nicotinic acid analogs studied, 2-pyrazinoic acid was the most potent inhibitor with

an apparent K_i of 0·075 mM. 2-Hydroxynicotinic acid was the second most potent with an apparent K_i of 0·23 mM; however, its structural congener, salicylic acid, had a slightly lower value of 0·16 mM (Fig. 1). Apparent K_i values for other analogs are listed in Table 1. Inactive as inhibitors at 0·5 mM were 2-Cl-, 2-Br-, 2-methyl-; 2,6-dihydroxy-, 2-hydroxymethyl-, 4-hydroxy-, 5-hydroxy-, 5-Cl-, and 6-hydroxynicotinic acid; 3-pyridylsulfonamide; nicotinamide; and ethyl-2-hydroxynicotinate.

TABLE 1. APPARENT INHIBITORY CONSTANTS (K_i) OF VARIOUS COMPETITIVE INHIBITORS OF NICOTINATE PHOSPHORIBOSYLTRANSFERASE IN HUMAN PLATELET LYSATE

Compound*	Apparent K_i (mM)
2-Pyrazinoic acid	0·075
Salicylic acid	0·16
2-Hydroxynicotinic acid	0·23
2-Fluoronicotinic acid	0·28
6-Chloronicotinic acid	0·56
Isonicotinic acid	0·75
3-Pyridylsulfonic acid	0·75
Pyridine	0·78
2-Aminonicotinic acid	0·82
Picolinic acid	1·16
3-Pyridylacetic acid	1·28
Benzoic acid	1·90

* Parallel studies were carried out at [^{14}C]nictotinic acid concentrations of 34, 17 and 6·8 μM in the presence and absence of the above compounds. See text for composition of incubation medium.

Nicotinate phosphoribosyltransferase catalyzes the first reaction in the pathway of Preiss and Handler for NAD biosynthesis.[5–15] The enzyme, in the main, is Mg^{2+}-dependent and, in some preparations, is stimulated allosterically by ATP.[14,16] Likewise, the velocity of the reaction catalyzed by this enzyme in human platelet lysate is stimulated by magnesium without alteration of the apparent K_m. Its apparent K_m of 24 μM agrees closely with that reported by Smith and Gholson[16] for partially purified enzyme from bovine liver. However, unlike the enzyme from bovine liver, neither the velocity nor K_m is changed by the addition of ATP.

The data relative to competitive inhibition by nicotinic acid analogs reveal marked specificity of the enzyme, since any substitution on the substrate greatly increases the apparent K_i relative to the apparent K_m. The same is true for purely isomeric changes as in picolinic and isonicotinic acids. Even so, general patterns based on chemical structure of the analogs emerge from the data.

Large substituents in the 2-position of nicotinic acid, e.g. $-NH_2$, -Cl, -Br, $-CH_3$, and $-CH_2OH$, result in compounds virtually devoid of inhibitory activity; therefore, data relative to this series of compounds could be explained on the basis of steric hindrance of the pyridine nitrogen. This hypothesis is further substantiated by the lack of inhibition by 2,6-dihydroxy- and 2,6-dichloro-nicotinic acid as well as the weak inhibitory capacity of 6-chloro-nicotinic acid. Positions 4 and 5 also appear vulnerable to steric effects, since 4-hydroxy-, 5-chloro-, and 5-hydroxynicotinic acids are not inhibitory at 0·5 mM. Such data are consistent with a size-restricted, hydrophobic region at the binding site of the enzyme to accomodate carbon atoms 4, 5, and perhaps 6 of the pyridine ring. This type of bonding was hypothesized by Mares-Guia and Shaw[17] for the active center of trypsin in which its substrate apparently was bound in a hydrophobic slit or crevice.

The keto-tautomer of 2-hydroxynicotinic acid is probably not the active form, since the apparent K_i of this compound is very close to that of 2-fluoronicotinic acid. The fluorinated species is of course incapable of this configuration.

The pyridine nitrogen of nicotinic acid is important for interaction with the enzyme for several reasons. Substitution of a carbon for this nitrogen atom, as in benzoic acid, greatly diminishes inhibitory activity. Substantial activity is retained, as in pyridine, even though the carboxyl group is deleted. Further support lies in the loss of activity in compounds where this nitrogen atom is sterically hindered and in the relatively large inhibitory capacity of 2-pyrazinoic acid.

A major difference, structurally, between benzoic acid, a poor inhibitor, and nicotinic acid is the unshared pair of electrons in the sp^2 orbital of the pyridine-nitrogen. Hence the profound divergence between the apparent K_i of benzoic acid and the apparent K_m of nicotinic acid may be explained on the basis of nucleophilic interaction of this sp^2 orbital with carbon-1 of PRPP. The relatively low apparent K_i values of 2-hydroxynicotinic acid and *o*-hydroxybenzoic acid (salicylic acid) lend further credence to this possibility. The position of these nucleophilic hydroxyl groups would also favor such interaction at the enzyme's catalytic site.

The carboxyl group of nicotinic acid is also important since its deletion, as in pyridine, diminished activity. Amidation, as in nicotinamide, and esterification, as in ethyl-2-hydroxynicotinate, result in compounds virtually devoid of inhibitory activity. The size of this acidic substituent is critical as well, since replacement by the larger sulfonic group, as in 3-pyridylsulfonic acid, resulted in an apparent K_i much higher than the apparent K_m of nicotinic acid.

The proximity of the carboxyl group to the pyridine ring is salient, since interposition of a methylene group, as in 3-pyridylacetic acid, results in a compound of weak inhibitory capacity. The positional relationship of the carboxyl group to the pyridyl-nitrogen is also meaningful, since isonicotinic and picolinic acids are quite weak inhibitors.

NAD, NADH, and NADP (0·5 mM) had no influence on the activity of nicotinate phosphoribosyltransferase in human platelet lysate. Such data are consistent with those reported by Imsande[8] for the enzyme derived from various bacteria and rat liver where feed back inhibition did not occur.

Although this discussion is mainly concerned with relating steric effects to the extent of inhibition, other possibilities remain. Resonance and induction influences of various substituents on the pyridine ring in general and of the pyridine nitrogen in particular could also be implicated in structure–activity relations.

In conclusion, the data suggest that the free carboxyl group, its position on the pyridine ring, and the pyridine nitrogen of nicotinic acid are particularly apposite to interaction with nicotinate phosphoribosyltransferase.

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