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SHORT COMMUNICATIONS

Epygid (2-ethyl-6-methyl-3-oxypyridine): a new agent for selective suppression of membrane-associated polypeptide chain translation in the brain

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2-Ethyl-6-methyl-3-oxypyridine (Epygid), a water-soluble, amphiphylic and low-toxic (LD_{50} = 900 mg/kg rat body weight) compound, synthesized for the first time by Dr L. D. Smirnov in 1980, possesses a marked antioxidant effect with some geroprotective action [1]. Subsequent studies performed by Burlakova *et al.* [2] have proven the ability of this substance to be incorporated in the lipid layer of biological membranes and due to this to change a number of their physicochemical properties. 100–300 mg per 1 g body weight are effective and safe doses for single i.p. injection to rats [2].

Modified membranes of endoplasmic reticulum obtained from brain cells of adult and old animals should be recognized as suitable models for elucidation of the role of membranes in regulation of protein synthesis being affected in aging.

It is known that in aging, during ontogenesis from 3 to 18 months in rats, the total content of polysomes decreases in brain from 2.2 ± 0.8 mg/g tissue ($P < 0.05$) to 1.0 ± 0.3 mg/g tissue ($P < 0.05$) which is caused by sharp destabilization of mRNA molecules [3]. Membrane-bound polysomes synthesizing many neuronspecific proteins account for only 20% of brain total polysomes in 3-month-old rats, but for 85% in 18-month-old animals [3]. The latter circumstance can testify to an increase of the functional role of membranes in aging.

The present paper describes the results of our experiments on the study of protein-synthesizing activity of polysomes, both free and bound to membranes of endoplasmic reticulum, isolated from brain of rats of different ages, subjected and non-subjected to single injection of membrane-tropic substance of Epygid.

Materials and methods

The drug at the doses of 150 and 300 mg per 1 kg of body weight was intraperitoneally injected to albino male rats of WAG/33Sto strain aged 3 and 18 months. Equal volume (0.6 ml) of physiological solution was injected to control animals. The animals were decapitated 1.5 hr after the injection. Brain tissue was homogenized in 3 vol. of the medium 0.05 M Tris–HCl (pH 7.40)/0.25 M sucrose/0.005 M $MgCl_2$/0.05 M KCl. Post-mitochondrial supernatant (S12) was isolated from homogenate due to centrifugation at 12,000 *g*, 15 min (+2°). S12 served as a source for isolation of fractions of free and membrane-bound polysomes according to Blobel and Potter [4] in Berman modification [5]. To obtain fractions of total polysomes before fractionation of S12 the latter was treated by Triton X-100 (final concentration of the detergent 2%, v/v), thus dissolving reticulum membranes [5]. Therefore, the obtained total polysomes were a mixture of initially free polysomes and polysomes separated from membranes by Triton X-100.

The amount of protein in polysome preparations and solubilized preparation of membranes was determined according to Lowry *et al.* [6].

Isolated preparations of polysomes were added to cell-free protein synthesis systems. To compose such systems we used Amersham Rabbit Reticulocyte Lysate Special Kit (ARRL, code No 90, Amersham, U.K.) for *in vitro* mRNA and polysomes translation in the presence of L-[4,5-^{3}H]leucine, TRK.510, 130–190 Ci/mmol (Amersham, U.K.). Preparation and work with these *in vitro* translation systems were performed according to Pelham and Jackson [7]. The total volume of incubation sample was equal to 0.335 ml. Polysomes were placed into the sample at the amount of 35 μg protein per sample. The samples were incubated for 1 hr at +37°. The reaction was discontinued by addition of equal volume of cold (+2°) 20% trichloracetic acid (TCA) containing 0.10% aqueous solution of unlabelled L-leucine. Acid-insoluble pellets were washed with 5% TCA (90°, 15 min), cooled to +4° and held in Synpor No. 6 membrane nitrocellulose filters with a pore diameter of 0.45 μ (Chemapol, Czechoslovakia). Radioactivity of filters was determined in toluol scintillation liquid using a SL4000 Scintillation Counter (Intertechnique, France). Incubation of parallel series of similar samples at 0° served as a special control for non-specific incorporation of [^{3}H]leucine into TCA-insoluble material.

For quantitative determination of Epygid content in membranes, polysomes were removed from membrane surface by treating with 0.025 M Tris–HCl pH 7.60, 0.02 M EDTA, 0.4 M KCl, then rapidly sedimented particles were precipitated through 0.5–2.0 M scalar (discontinuous) sucrose gradient at 280,000 *g*, 3.5 hr (+2°) in Spinco LS-85 centrifuge, rotor 85 Ti [5]. Membrane fraction concentrated in gradient interphase was treated with Triton X-100 to the final concentration of 2% (v/v). Then the mixture was subjected to SDS-water/benzol extraction according to Kuznetsov and Zherdev [3], water phase was collected and quantitative determination of Epygid was performed by special HPLC procedure [3]. The determination was carried out in Altex/Tracor 920A chromatographic system with quantitative evaluation of fractions by Chromatopac C-R1A integrator (Shimadzu, Japan). The main parameters of chromatographic routine: Alltech C-6000 column (4.6 × 25 mm), stationary phase—S5SN-ODS, liquid phase (eluent)—methanol : water (1 : 1, v/v), flow rate—1.0 ml/min, pressure in column—2000 p.s.i., temperature in column—+25°, retention time of the studied substance—8.38 min. The final identification of Epygid extracted from biological material was performed by chromatographically pure standards both chromatographically and mass-spectrometrically according to the technique described earlier [3].

Results and discussion

As seen from our data (Table 1), protein-synthesizing activity of polysomes of both types somewhat increases with the age of the animals. Since in our experiments exogenous polysomes were placed in standard conditions, not imitating "aged surrounding" of polysomes *in situ*, it is not possible to interpret this fact in one way. However, increase of translation rate in aging *in vivo* has been already observed by Khasigov *et al.* [8]. Increase of translation rate, being difficult to control, in combination with mRNA destabilization and prolongation of existence (t_i) of a number of cellular proteins in many ways determine biochemical

Table 1. The activity of rabbit reticulocyte lysate cell-free translation system in the presence of different polysomal populations isolated from total brain of adult and old rats after a single I.P. injection of 2-ethyl-6-methyl-3-oxypyridine (Epygid)

Age of animals (months)	Drug dosage (mg/kg body weight)	Drug content in membranes, (ng/mg protein) (M ± S.E.M.)	Incorporation of L-[^{3}H]leucine into *in vitro* synthesized products during 1 hr incubation in the presence of different types of polysomes, c.p.m./mg protein (M ± S.E.M.)		
			Free	Membrane-bound	Total
3	0	—	17863 ± 321	12287 ± 189*	18117 ± 289*
3	150	88 ± 7	17087 ± 243	9037 ± 92*	18290 ± 189
3	300	196 ± 11	19035 ± 140	4119 ± 98	17981 ± 135
18	0	—	21999 ± 387	34478 ± 311	21827 ± 271
18	150	103 ± 9	21320 ± 316	16609 ± 293	21877 ± 374
18	300	218 ± 14	22634 ± 281	6428 ± 74*	21768 ± 258

* $P < 0.01$, for all other data in the table $P < 0.05$. Mean data of 8 experiments are presented. Five animals were taken for each experimental point (for polysome isolation).

picture of aging [8, 9]. Inadequate "senile" enhancement of translation serves as one of the causes of formation and accumulation of errors in primary structure of translated proteins [8, 9].

Application of water-soluble amphiphylic membranetropic chemical agents may appear one of the ways to control a number of metabolic disturbances of old age occurring at the level of biological membranes as carriers of protein-synthesizing apparatus.

We have confirmed earlier that Epygid is incorporated in the content of endoplasmic reticulum membranes of rat brain cells not transforming into any possible metabolites, and that maximal content of the substance in membrane is achieved 1–1.5 hr after single i.p. injection [3]. As seen from Table 1, injection of the substance does not effect the translating activity of free and total, i.e. torn off membranes, polysomes. At the same time the rate of assembling of polypeptide chains on membrane-bound polysomes is very sharply slowed down under the effect of Epygid, and this slowing down is directly dependent on the content of the substance in membrane. It should be noted that cellular membranes of old animals incorporate this substance by about 15% more as compared to cellular membranes of young organisms. This is, possibly, associated with the fact that in injection of equal amounts of the substance to old and young animals significantly more marked slowing down of translation occurs on membranes isolated from cells of old animals (see Table 1). These data permit us to suppose that even 15% predominance of Epygid incorporation in membranes of "old" cells as compared to "young" ones causes such an essential additional transposition of infrastructure of membrane lipid layer and as a result, of all membrane in general, that suppression of protein formation by polysomes on these membranes is significantly higher by its intensity than simple 15% enhancement of translation inhibition.

In the course of membrane isolation for determination of Epygid in their content, we proved that the preparation does not change the amount of polysomes localized on these membranes. So, the amount of protein we determined [6] in RNP particles separated from membranes by treatment with EDTA and KCl (see above) was absolutely independent of the fact that whether the preparation was injected to the animal or not. For 3-month-old rats this was equal to 0.25 ± 0.072 mg protein per 1 g tissue ($P < 0.05$); for 18-month-old rats this amount accounted for 0.410 ± 0.089 mg/g ($P < 0.05$).

At the final step of present study we have investigated a direct effect of Epygid on cell-free translation by two types of polysomes (free, bound) after the *in vitro* drug addition (Table 2). These data are indicates that only a membrane-bound polysomes are sensitive to Epygid presence.

Thus, we for the first time detected Epygid ability to effectively and selectively slow down translation occurring only on brain cell membrane-bound polysomes. We hope that further molecular pharmacological study of this substance and its analogs will lead to creation of a new class of membrane-active inhibitors of translation, i.e. drugs selectively accomplishing their effect only at the level of membrane-bound polysomes.

Table 2. Effect of Epygid on protein synthesis promoted by 3-month-old rat brain free and membrane-bound polysomes in special *in vitro* experiments with direct drug addition into cell-free translation systems*

Epygid content (μmol/ml)	Translation activity, c.p.m./mg protein (M ± S.E.M.)	
	Membrane-bound polysomes	Free polysomes
1.0	12785 ± 368	16431 ± 622
5.0	10889 ± 219	15990 ± 527
10.0	7836 ± 167	16720 ± 499
25.0	4662 ± 122	17321 ± 603
50.0	2459 ± 101	16808 ± 512
100.0	931 ± 81	15939 ± 493
None (control)	16883 ± 697	16883 ± 697

* Mean data of 9 experiments are presented, for each experimental point $P < 0.05$.

Use of cell-free translation systems in such studies should be recognized as a fruitful approach to investigation of molecular effects of oxypyridines at the level of biomembranes.

In summary, intraperitoneal injection of different amounts of Epygid-(2-ethyl-6-methyl-3-oxypyridine) to 3- and 18-month-old rats led to significant reduction of translating activity *in vitro* of membrane-bound polysomes of rat brain cells, but not of free polysomes. This regularity is more marked in case of endoplasmic membranes of 18-month-old animals than those of 3-month-old. Separation of polysomes from membranes by Triton X-100 resulted in restoration of template activity of the former to the level of free polysomes. It has been proven that the observed phenomenon is associated with incorporation of Epygid into the composition of membranes of endoplasmic reticulum which contain a part of cell polymers on their surface.

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Effect of the cholangiographic agent, ioglycamide, on the β-glucuronidase activity in rat liver and bile: relevance with regard to bilirubin deconjugation

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A fraction of bilirubin conjugated in the liver subsequently undergoes deconjugation [1–3]. In the rat, this fraction amounts to approximately 6–7% of the administered load of bilirubin mono- and diglucuronides [2, 3]. The mechanism of this process is unknown. Involvement of β-glucuronidase (EC 3.2.1.31) is suggested by the observations that the enzyme is abundantly present in liver tissue [4] and that its activity is associated with bilirubin granules in cholestatic liver tissue [1].

In rats, biliary excretion of the iodinated cholangiographic agent ioglycamide (the meglucamine salt of ioglycamic acid, Biligram®, Schering A.G., Berlin, F.R.G.) induced a marked choleresis together with a decrease in the biliary output of bilirubin conjugates [5]. In addition, ioglycamide treatment also led to a significant increase in the diconjugated to monoconjugated bilirubin ratio in both bile and serum [5]. The question thus arose as to whether deconjugation of bilirubin diglucuronide could be decreased by ioglycamide. Indeed, iodinated contrast agents inhibit the activity of purified β-glucuronidase presumably by non-specific binding to the enzyme [6]. We therefore investigated whether infusion of ioglycamide into rats decreases the activity of β-glucuronidase in bile and/or liver tissue, and whether this could in turn lead to decreased hydrolysis of conjugated bilirubins.

Male Wistar rats (330–345 g body wt) provided with a biliary cannula were infused via a jugular vein catheter with glucose 5% (w/v) in saline. After 18–20 hr, two 20-min basal bile samples were collected and rats were then infused for 70 min with ioglycamide (5.5 μmol/min/kg body wt), a load saturating its hepatic transport capacity (Tm) [7]. After a 50-min equilibration period, a 20-min bile sample was collected in the dark, into a pre-weighed plastic tube cooled on ice. Control rats received saline alone.

At the end of the 70 min infusion period, animals were killed and their liver rapidly removed and homogenized in ice-cold 0.25 M sucrose, pH 7.4 containing 1 mM disodium EDTA. Homogenates were centrifuged to separate nuclei, unbroken cells, and tissue debris (pellet or N-fraction) from other organelles and cell sap (supernatant or E-fraction) [8]. Studies were performed on aliquots of bile and N- and E-fractions. In some experiments, the E-fraction was further separated into four additional fractions by ultracentrifugation [8], in order to investigate the subcellular distribution of the β-glucuronidase activity.

Bile volume was determined gravimetrically without correction for specific gravity. The concentration of bilirubin conjugates in bile was measured after diazo-coupling with ethyl anthranilate [9]. β-glucuronidase activity (EC 3.2.1.31) was assayed according to Gianetto and De Duve [10]. Other enzyme activities (their putative subcellular localisation is given in brackets) were determined by established methods: lactate dehydrogenase (cytosol) EC 1.1.1.27 [11], 5′-nucleotidase (plasma membrane) EC 3.1.3.5 [12]. Activities are expressed as μmol substrate hydrolysed per hour. Values per g total liver weight were