

Human Skin Fibrosis RNase Search for a Biological Inhibitor-Regulator

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Zusammenfassung

Eine beim Menschen auftretende, durch Röntgenbestrahlung verursachte Hautfibrose enthält äußerst aktive Ribonuclease (RNase). Dies steht wahrscheinlich mit dem Auftreten einer Fibrose nach der Röntgenbestrahlung in Zusammenhang. Wir haben einen extrem gereinigten Extrakt aus goldenen Blättern des *Ginkgo biloba* hergestellt, der als biologischer Regulator fungiert. Er normalisiert in großem Maße die übermäßig starke RNase-Aktivität in einem Extrakt aus bestrahlten menschlichen Hautzellen, beeinflusst jedoch nicht die Aktivität der normalen menschlichen Plasma-RNase. Dieser Extrakt kann zur Fibrosebehandlung verwendet werden.

Schlüsselwörter

Krebs, Bestrahlung, Hautfibrose, Ribonuclease, Primer, *Ginkgo biloba*.

Summary

Human skin fibrosis caused by radiotherapy contains very active ribonucleases (RNases). This is probably connected with the appearance of postradiotherapy fibrosis. We prepared a highly purified extract of *Ginkgo biloba* golden leaves, which behaves as biological regulator. It normalizes to a large extent the excessive RNase activity in an extract of irradiated human skin cells, but does not affect activity of normal human plasma RNase. This extract may be used for this fibrosis treatment.

Keywords

Cancer, irradiation, skin fibrosis, ribonucleases, primer, *Ginkgo biloba*.

Blood plasma of most cancer patients contains RNases which exhibit a highly increased hydrolytic

activity in the presence of RNA(s) [1], [2], thus contributing to the decrease of homeostasis. In the plasma of cancer patients, these enzymes may extensively degrade oligoribonucleotides which play an essential role in priming DNA replication in normal cells [3], [4] and in regulating protein biosynthesis at the ribosomal level [5]. One of us (*M. Beljanski*) searched for a biological regulator which could restore to normal this pathologically increased plasma RNase activity. As irradiated skin cells also exhibit excessive RNase activity, patients suffering from radiotherapy-induced skin fibrosis might be expected to benefit from such a regulator.

We show here that a fraction isolated and purified from soluble extracts of *Ginkgo biloba* golden leaves efficiently regulates in vitro the pathologically increased RNase activity of cancer patient plasma and of fibrosic skin.

Material and Methods

Unlabelled and [³H]-labelled ribosomal RNA(s) (r-RNA) and transfer RNA(s) (t-RNA) were isolated and purified from extracts of *Escherichia coli* as previously described [6]. RNA yield was determined by measurement at 260 nm. The RNA purity (260/280:2.0-2.1); and integrity were checked using polyacrylamide gel electrophoresis. RNA radioactivity was measured with a Packard (Prias) liquid scintillation counter. Whatman GF/C glass filter from Whatman Ltd, Maidstone, England. Trichloroacetic acid (TCA): Prolabo, France. Plasma was obtained from peripheral blood collected in the presence of complexon (EDTA) and centrifuged at 6000 rpm. for 5 minutes. Storing plasma at -20°C does

not modify RNase activity. Proteins were measured using the Biuret method [7]. Skin cells from radiotherapy induced skin fibrosis patients were supplied by the Oncology Department, Avicenne Hospital, Bobigny.

Bioparyl Preparation

Golden leaves of the *Ginkgo biloba* tree were extracted with boiling water for several hours. Water extract was concentrated by evaporation and hydrolysed with HCl (final concentration) at 100°C for 20 minutes. After partial neutralization the mixture was first centrifuged, then the supernatant was fractionated on a Sephadex G-25 fine column and eluted with water. Only one fraction constitutes Bioparyl [8].

IR spectroscopy, mass spectrometry and chromatography were used to identify the essential components.

The infra-red spectrum was obtained with a Perkin-Elmer spectroscope (157w11007).

For mass spectrometry, the compounds were sampled as solutions of 0.1 g/l in methanol, which is the best solvent. All the samples examined were introduced directly into the source with an appropriate probe according to the usual procedure. All spectra were obtained using a Nermag R-10-10-H spectrometer (Rueil Malmaison, France), with a combined CI, EI source, probe with fine wire (desorption) and small cup (direct inlet). The reagents employed for the CI spectra were methane N30, isobutane N25 and ammonia N36 supplied by Air Liquide (division scientifique Alphagaz France).

EI mass spectra were obtained at 70 eV with a source temperature of 130°C. The source chamber was maintained at 4.4×10^{-7} Torr, as measured with an ionization gauge. The CI spectra were determined using 83 eV to ionize the reagent gases at 130°C. The source chamber was maintained at 1×10^{-5} Torr controlled the source pressure at about 1 Torr for the three gases. In all cases, DCI and DEI spectra were obtained by desorption of a rhenium wire, produced by an emission current varying from 50 mA to 1 A at 0.1 A/s. Direct inlet spectra, with small cups, were also obtained. All spectra were obtained at maximum total ionic current and reproducibility reached at least 91%.

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For the assay of RNase activity, incubation mixture (0.15 ml final volume) contained Tris-HCl buffer, pH 7.50, 25 μ Mol; [3 H] - RNA (100 μ g):20000 counts per minute (cpm); plasma proteins and/or Bioparyl as indicated in the legends to the figures. After incubation of the mixture at 36°C for 10 min., TCA precipitable labelled material was washed three times with 10 ml of a 5% TCA solution on a glass millipore filter GF/C, then with 95° alcohol. After drying, radioactivity was measured. Results are expressed as percent of undegraded [3 H]-RNA/assay.

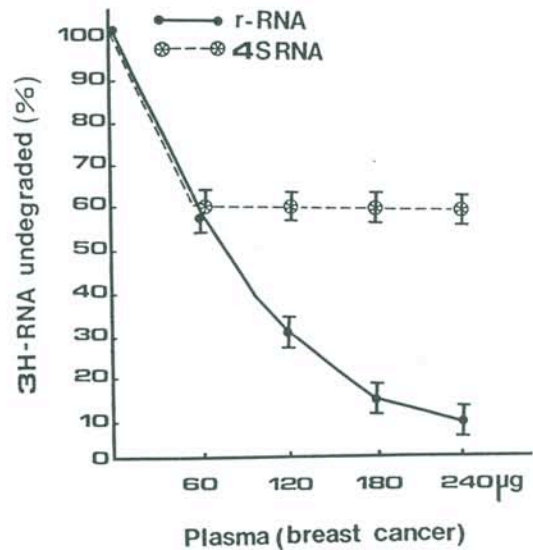
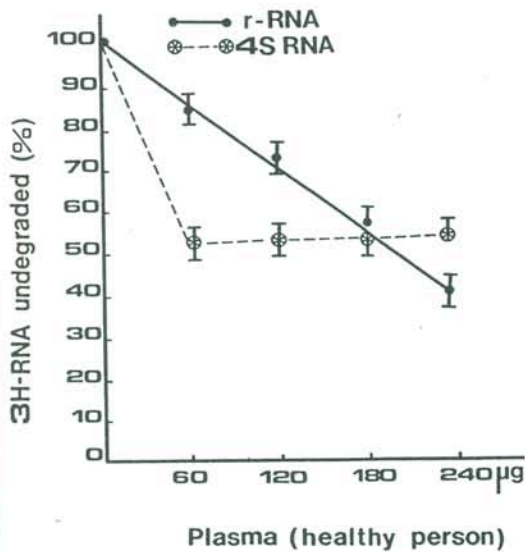
Results and Discussion

The IR absorption peaks of the extract show aromatic vibrations (1610 cm^{-1}) and the presence of C-O bonds.

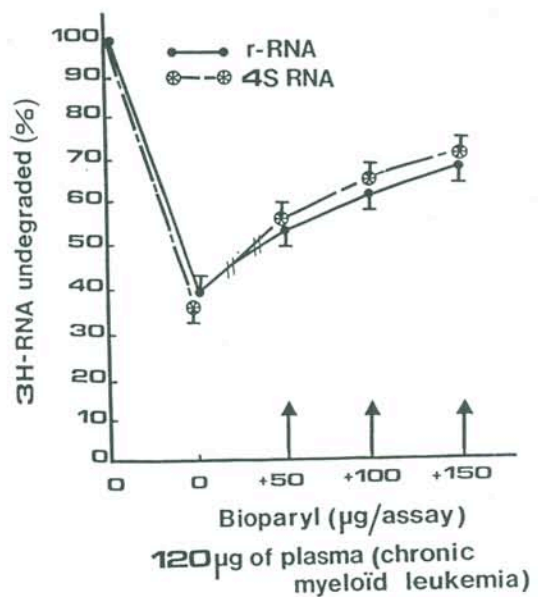
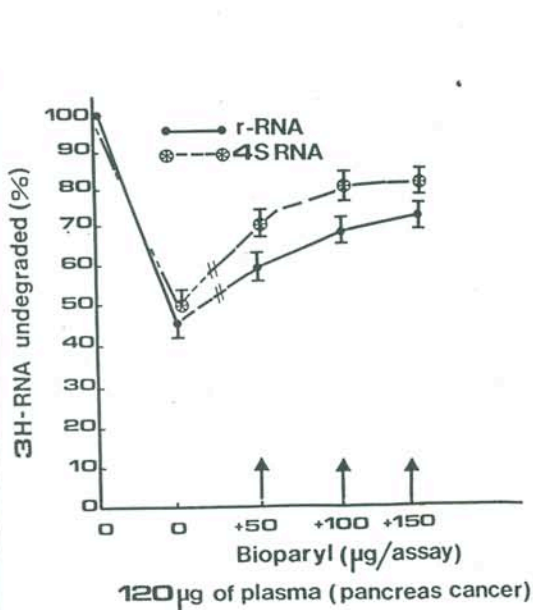
The ammonia CI mass spectra show both [M+1] and [M+18]⁺ for two compounds of m/z 350 and 440 respectively.

Mammalian plasma RNase is similar

to pancreatic RNase (E.C 3.1.27.5) in spite of its different origin. Plasma RNases constantly interact with a large number of different molecules and exhibit varying activities, particularly under pathological conditions. Fig. 1 shows that plasma RNases of healthy controls differentially degrade [3 H]-rRNA (2000-3500 nucleotides) and [3 H]-transfer RNAs (75 nucleotides).



Figs. 1 and 2: RNase activity in human healthy and breast cancer plasma (means \pm S.D; n=4). Incubation conditions (see text).



Figs. 3 and 4: Effect of Bioparyl on RNase activity in human pancreas cancer and chronic myeloid leukemia plasma. (means \pm S.D; n=4). Incubation conditions (see text).

Under the same experimental conditions, [^3H]-r-RNA is much more extensively degraded by plasma RNases of breast cancer patients than by those of healthy controls (fig. 2). Yet, surprisingly, [^3H]-4S RNA is degraded to the same extent by plasmas of cancer patients and of healthy controls, suggesting the presence of two plasma RNases, one specific to long chain RNAs and the other specific to short chain RNAs.

Bioparyl inhibits degradation of both [^3H]-r-RNAs and [^3H]-4S RNAs by plasma enzymes of cancer patients (fig. 3 and 4), but not by those of healthy controls (fig. 5). Inhibition varies to a small extent according to types of cancer (and/or to individual patients). Figs. 3, 4 and 6 show that Bioparyl is capable of restoring RNase activities close to normal.

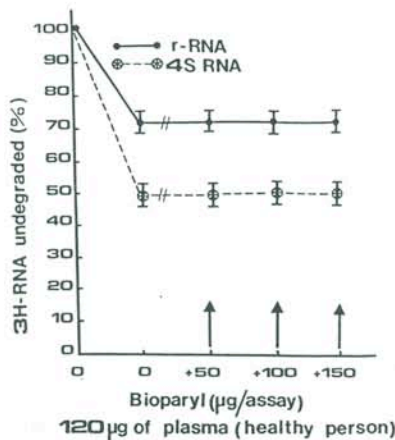
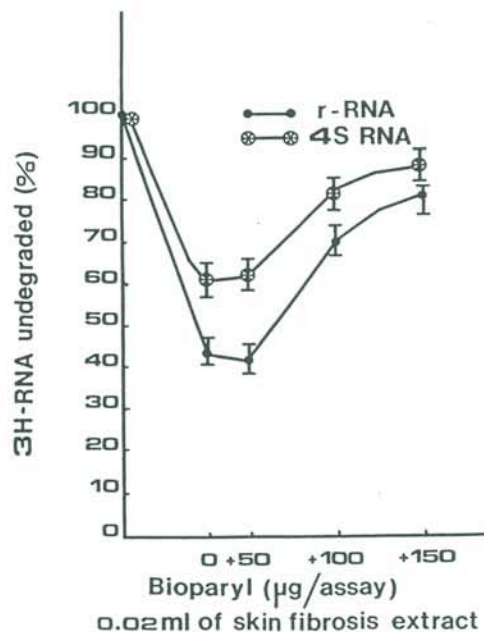
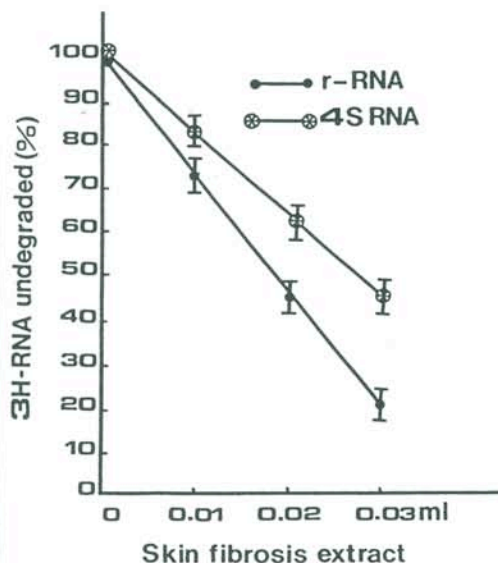


Fig. 5: Effect of Bioparyl on RNase activity in healthy human plasma (means \pm S.D.; n=4). Incubation conditions (see text).

RNases derived from skin fibrosis extract also exhibit substantially increased hydrolytic activities affecting both [^3H]-r-RNAs and [^3H]-4S RNAs (fig. 6). Bioparyl inhibits to a large extent enzyme activity in the

presence of each of these substrates. This potentially important observation suggests that radiotherapy induced skin fibrosis could be attenuated or even suppressed in vivo by a biological regulator or protector such as Bioparyl, which by controlling RNase activity could regulate the production of various RNAs, comprising messenger RNAs as well as short chain primers for DNA replication and transcription.

Previous research has shown by one of us (M.B.) that cancer DNA is a destabilized molecule ([9]) and is thus liable to exaggerated transcription into messenger RNA, leading to excessive production of various proteins and notably enzymes. This process may be progressively slowed down by Bioparyl, which in addition to its direct effect on RNases also binds to DNA and to RNA.



Figs. 6a + 6b: RNase activity in human skin fibrosis extract (a).

Effect of Bioparyl on RNase activity in human skin fibrosis extract (b). (means \pm S.D.; n=4). Incubation conditions (see text).

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