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**DNA MODIFICATIONS INDUCED BY IN VITRO IRRADIATION IN ASSOCIATION WITH AN ANTIBIOTIC CHEMICAL AGENT - NETROPSIN**

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Abstract

Netropsin, an antitumor/antiviral antibiotic, binds to cellular DNA in the minor groove of the double helix. Minor groove drugs have the ability to act as (i) DNA sequence recognition agents and (ii) modifiers of protein-DNA function. The use of antitumoral antibiotics in the radiotherapy of tumor determines a special interest in the behavior of these ligands during the irradiation of the cell. The present study has investigated the influence of in vitro netropsin treatment on apoptosis induction in normal human lymphocytes and also on the radiosensitivity response of this type of cells. The parameters used to characterize the cellular response of lymphocytes to irradiation were the micronucleus induction and the apoptotic response. The experimental results have revealed that netropsin presence in lymphocytes culture media does not produce any significant effect on apoptotic induction, at least for incubation of less than 48h, at about 10 µM Nt concentration. For these treatment conditions the netropsin has no influence also on the level of radioinduced lymphocyte apoptosis observed after 2 Gy gamma irradiation. After gamma irradiation only the micronucleus induction seems to be, however, modified by the netropsin treatment, the antibiotic acting as a radiosensitising agent at 10 µM concentration.

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Introduction

Molecular studies have revealed that some antitumor/antiviral antibiotics such as berenil, distamycin and netropsin (Fig.1) bind to cellular DNA in the minor groove of the double helix [1]. It is believed that netropsin (Nt) binds to A+T rich regions within the minor groove but do not intercalate between base pairs. 17 water molecules would be released per bound netropsin molecule in the minor groove. The antibiotic molecule displaces the spine of hydration within the minor groove, without unwinding or elongating the double helix but having as an effect a widening of the groove and a slight bending of the helix axis [2].

![Netropsin chemical structure](image)

**Fig.1 – Netropsin chemical structure**

Netropsin, likewise berenil and distamicin, binds preferentially to clusters which contain at least four A-T base pairs. As a consequence, these DNA regions are protected from different nuclease digestion [1].
Netropsin was found to inhibit also the activity of topoisomerases. At the level of cell physiology it disrupts the cell cycle, prolonging G1 and arresting in G2 [3].

Minor groove drugs have the ability to act as: (i) DNA sequence recognition agents and (ii) modifiers of protein-DNA function. They are also extensively studied because of their potent anti-tumor activity. Novel synthesized anticancer agents, having a netropsin-like moiety, were shown to inhibit topoisomerases I and II, to block the cell cycle at the G2-M phase and to induce apoptosis exhibiting remarkable anti-tumor activity against various xenografts [4].

The use of antitumoral antibiotics in the radiotherapy of tumor determines a special interest in the behavior of these ligands during the -irradiation of the cell. DNA ligands can increase or decrease the radiation damage of the cell [5, 6]. It was shown that free ligands in the irradiated solution can protect the DNA molecule against radiation damage. In contrast, the ligands bound by intercalation does not exhibit this ability.

In some experimental trials performed in the group of Dr. Spotheim-Maurizot [unpublished results – from Center of Molecular Biophysics, CNRS, Orleans] concerning the netropsin effect on DNA radiolysis it was found that at concentration higher than 50 µM Nt acts as a radioprotector and at concentration of about 10 µM as a radiosensitizing agent. It is important however to mention that the drug concentrations used for such molecular studies are much higher than the concentrations found cytotoxic (about 1 µM) in experiments performed on living cells [4].

In the present study we have investigated the influence of in vitro netropsin treatment of human lymphocytes on apoptosis induction. We also tried to evidence a possible influence of netropsin incubation on cell radiosensitivity. The parameters used to characterize the cellular response of lymphocytes to irradiation were the micronucleus induction and the apoptotic response.

Materials and methods

Cell culture and incubation

Netropsin (SIGMA) stock solutions of 4 mM were prepared in 10 mM NaCl in 10 mM Tris/HCl pH 7.5. Further solution for lymphocyte incubation was made in RPM1 culture medium at concentrations in the range of 1-20 µM. Heparinized blood samples were drawn by venepuncture from healthy volunteers; peripheral blood mononuclear cells (PBMc) were isolated by density gradient centrifugation on Ficall-Hypaque (Lymphoprep, Life Technology, Merelbeke, Belgium) and then washed and re-suspended in RPM1 1640 (Sigma) supplemented with 12% fetal calf serum (FCS). The lymphocytes have been treated with netropsin in either G0 cultures or phytohaemagglutinin (PHA) stimulated cultures. For the G0 incubations, the lymphocytes were transferred in plates with wells at a density of ~ 10^6 cells/ml/cm^2 and kept for time periods of 24-72 h. To induce cell proliferation, lymphocytes were suspended in growth medium supplemented with PHA 1 µl PHA/ml (SIGMA) medium at a cell density of 10^6 cells/ml.

Irradiation procedure

The PBMC were gamma irradiated in a ^{60}Co beam at a dose-rate of 2 Gy/min at room temperature for a total dose of 2 Gy. Irradiation was usually performed on cells either previously maintained for 20h in the G0 state (with or without netropsin incubation) or stimulated for 20h
with PHA (with or without netropsin treatment of 2h before irradiation). Just before the irradiation, the cells were re-suspended in a simple RPM/medium without adding any other component (FCS, PHA or netropsin). After exposure to gamma irradiation, the cells were re-suspended and kept in culture media for 24h under the same conditions of incubation as before the moment of irradiation.

Analysis of apoptosis induction

The apoptosis induction has been studied on lymphocytes kept in G₀ state for 24-72 hours or on PHA stimulated cells, in correlation with the netropsin treatment and with the irradiation treatment. The method used was the fluorescence staining and the morphological characterization of cells after analysis in a fluorescence microscope [7]. Briefly, the lymphocytes in suspension (≈10⁶) were mixed with a staining solution (1:1) containing 20 µg/ml acridine orange (AO) (SIGMA) and 10 µg/ml ethidium homo-dimer-1 (EthD-1) (Molecular Probes Inc) in saline phosphate buffer (PBS).

The cells were stained in the dark for 10 min, then centrifuged and resuspended in 1% formaldehyde in PBS. About 50 µl of the cell suspension were displayed on slides and scored under the fluorescence microscope. Apoptotic and viable cells with non-permeable cell membranes were stained green-yellow by AO, while apoptotic cells with permeable membranes and necrotic cells were stained orange-red by AO and EthD-1. The classification of cells included apoptotic cells (early or late apoptotic), with or without permeable cell membrane, and viable cells, which are recognized by the typical morphological description of the nucleus and the cell membranes reported in the literature [7]. At least 200 cells were scored for every sample analysis.

Micronucleus assay

The PBMC cultures stimulated with PHA were kept for 72 h in a CO₂ incubator and cytochalasin-B (4 µg/ml, SIGMA) was added after 40h of culture. At the end of the culture period the cells were centrifuged, given a hypotonic shock (75 mM KCl) and fixed 2 times in methanol: acetic acid (10:1) [8]. After fixation cells were dropped on slides and stained with Giemsa for 20 min. To evaluate the micronucleus induction, 500 bi-nucleated cells were scored per sample. MN were identified according to the criteria summarized by Fenech [9].

Results and Discussions

Influence of netropsin incubation on apoptosis induction in lymphocytes

The influence of netropsin incubation on apoptosis induction was tested for lymphocytes maintained either in G₀ state or stimulated to proliferate, with PHA. The percentages of viable, early apoptotic or late apoptotic lymphocytes found after different time intervals of incubation with netropsin in the low concentration range (1-20 µM Nt) are presented in Table1. For the lymphocytes kept in G₀ stage for 24h and 48h, the incubation with netropsin does not produce any effect on apoptosis induction, for any of the used netropsin concentration. However, for 72h treatment with netropsin some higher early apoptosis level is observed (20%) in comparison with the non-treated lymphocytes (14%) kept 72h in G₀ culture.

A similar tendency of modification is observed for the PHA stimulated lymphocytes after 72h incubation in 20 µM Nt. It is interesting to notice that the PHA stimulation, by itself, is producing a higher apoptosis induction comparative with the cells maintained in G₀ cultures, fact already described in the literature [7].
Influence of Netropsin incubation on the radiosensitivity of human lymphocytes

The radiosensitivity of the separated lymphocytes was studied by observing the micronucleus induction and the apoptosis induction consecutive to 2Gy in vitro gamma irradiation, with or without concomitant netropsin treatment. The results are presented in Table 2 (A and B). In the case of the cultures for MN induction, the netropsin treatment was performed at a concentration of 10 µM Nt, in the G0 state of the cells, for a time interval of 20h, before irradiation, at 37°C and under CO2 atmosphere. Immediately after irradiation, the cells were stimulated to proliferate by PHA addition, the culture medium being supplemented also with netropsin at the same concentration (10 µM Nt) as before.

The micronucleus yield due to 2 Gy irradiation was found to be further increased by the netropsin treatment (Table 2A). The increase is highly significant and suggests a radiosensitizing influence of netropsin at this range of concentration. In fact, in experiments at DNA molecular level, concerning the influence of netropsin on the radiolysis of AG30 plasmide, it had been observed that netropsin has a radioprotective effect at concentrations higher than 200 µM. For a lower range of concentration (between 10 to 20 µM) an opposite effect was observed, netropsin acting like a radiosensitising agent. The protecting effect towards DNA radylisis was observed only for a binding ratio superior to 0.1 netropsin molecule per DNA base pair [Center of Molecular Biophysics, CNRS, Orleans - unpublished results].

Our results at cellular level are confirming the data reported at molecular level, assuming a radiosensitising effect of netropsin at 10 µM concentration. The radioinduced apoptosis of cells seems not to be influenced by the previous incubation of cells in 10 µM Nt (Table 2B). The percentages of the apoptotic cells have been determined 24h after irradiation. The observation is valid for both netropsin incubation in G0 state and after PHA stimulation.

Table 2. Influence of netropsin incubation on the radiosensitivity of human lymphocytes: (A) MN induction; (B) Apoptosis induction
(A) MN induction – assay at 72h after PHA stimulation

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Nonirradiated MN per 1000 BN cells</th>
<th>% BN cells with</th>
<th>2 Gy gamma irradiation at 20h after the start of cultures</th>
<th>%BN cells with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0MN 1MN 2MN 3MN</td>
<td>0MN 1MN 2MN 3MN</td>
<td></td>
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<tr>
<td>Control lymphocytes</td>
<td>25±1 98±5 1.5±0.1 0.5±0.1 -</td>
<td>282±14 75.3±4 21±1 3.6±0.2 -</td>
<td></td>
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</tr>
<tr>
<td>Lymphocytes +10 µM Nt, 20h incubation before PHA stimulation</td>
<td>32±2 97±5 2.6±0.1 0.3±0.02 -</td>
<td>395±20 66.5±3 28±1 5±0.3 0.5±0.1 -</td>
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(B) Apoptosis induction

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Nonirradiated Viable cells Early apopt. Late apopt. Total apopt.</th>
<th>2 Gy gamma irradiation at 20h after the start of cultures Viable cells Early apopt. Late apopt. Total apopt. Radiation induced apopt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0 culture 44h Control lymphocytes</td>
<td>85±4 8.6±0.4 6.5±0.3 15±0.8</td>
<td>71±4 17±1 12±0.6 29±2 14±1</td>
</tr>
<tr>
<td>G0 culture 44h lymphocytes+ 10µM Nt, incubation 44h</td>
<td>88.5±4 8.7±0.4 2.7±0.1 11.4±1</td>
<td>73.5±4 18.2±1 8.2±0.4 26.4±1 15±1</td>
</tr>
<tr>
<td>PHA stimulated culture 44h Control lymphocytes</td>
<td>78.5±4 3.7±0.2 17.7±1 21.4±1</td>
<td>73.2±4 7.7±0.4 19±1 26.7±1 5.3±0.3</td>
</tr>
<tr>
<td>PHA stimulated culture 44h lymphocytes +10 µM Nt, incubation the last 26h of the culture</td>
<td>83.5±4 4.2±0.2 12.2±1 16.4±1</td>
<td>78.2±4 9.0±1 12.7±1 21.7±1 5.3±0.3</td>
</tr>
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</table>

Conclusion

The reported experiments have shown that:

- the netropsin presence in lymphocytes culture media does not produce any significant effect on apoptotic induction, at least for incubation of less than 48h, at about 10 µM Nt concentration.
for the above treatment conditions the netropsin has not influence on the the level of apoptosis radioinduced in lymphocyte cells by 2 Gy gamma irradiation.

the micronucleus induction due to gamma irradiation seems to be, however, modified by the netropsin treatment, the antibiotic acting as a radiosensitising agent at 10 \( \mu \text{M} \) concentration. For that range of concentrations, the binding of netropsin in the minor groove of DNA does not offer any protection against the DNA breakage induced by radiation.

References