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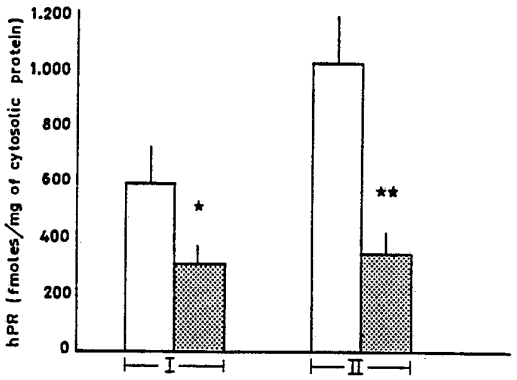
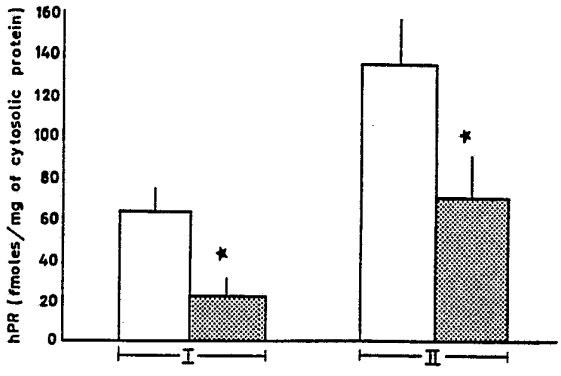
(54) Title: ANTISENSE OLIGONUCLEOTIDES

(57) Abstract

The present invention is directed to an antisense oligonucleotide comprising a nucleotide sequence which is characterized by its ability to hybridize under stringent hybridization conditions to a part of the mRNA sequence of the human progesterone receptor and further characterized by its ability to block the synthesis of the human progesterone receptor, and modifications thereof, to a process for the preparation of such antisense oligonucleotides and modifications, pharmaceutical compositions containing such antisense oligonucleotides and modifications and the use of these antisense oligonucleotides and modifications as a therapeutic agent.





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Antisense oligonucleotides

The present invention is directed to antisense oligonucleotides, specifically to antisense oligonucleotides blocking the synthesis of the human progesterone receptor (hPR).

The traditional obstacle in the identification of molecules having agonistic or antagonistic activity regarding specific human steroid hormones is based on the structural similarities of the corresponding receptors. These are in fact members of a family of proteins most likely evolved from a single ancestral gene. All of these intracellular receptors show 3 typical functional domains, namely the amino-terminal domain which is most likely involved in the interactions between the receptor protein and transcription factors, the DNA-binding domain, located in the internal portion of the protein and the carboxy-terminal domain, responsible for the ligand recognition. It is known in the art that for example, none of the molecules synthesized today showing high affinity for the hPR are devoid of any side activity on the human glucocorticoid receptor [Goodmann and Gilman's "The Pharmacological Basis of Therapeutics", 8th edition, Pergamon Press, New York 1990].

There is, however, a considerable interest in specific antagonists of the hPR, e.g. for medical purposes, e.g. for the termination of a pregnancy. According to Goodman and Gilman (see above) antiprogestativa in general act in the following manner. When administered to normal women in the follicular phase of the menstrual cycle, they prevent ovulation by blocking progesterone action at the level of pituitary or hypothalamus: this suppresses the midcycle surge of gonadotropins and delays follicular development. During the luteal phase of the cycle, they act directly on the uterus to block the

action of progesterone, resulting in release of prostaglandins from the endometrium and subsequent menstrual bleeding. Similarly, they can terminate early pregnancy by facilitating luteolysis, menstruation, uterine motility, and detachment of the embryo.

In view of such interest in the development of antiprogestativa, and because of the difficulties encountered in the development so far it is advisable to envision alternative strategies in the design of such a specific drug. One possibility is to block hPR synthesis post-transcriptionally by the presence of high concentrations of "antisense oligonucleotides" (aON). The aONs are designed to recognize a specific sequence of the mRNA of the hPR and to block the synthesis of the hPR.

Such an approach has already been used for inhibiting the synthesis of receptors like the nicotinic acetylcholine receptor, the β_1- and β_2 adrenergic receptors and the glycine receptor and the retinoid receptor [Sumikawa and Miledi, PNAS 85, 1302-1306 (1988); Guest et al., J. Biol. Chem. 265, 5370-5375 (1990); Akagi et al., PNAS 86, 8103-8107 (1989); Cope and Wille, PNAS 86, 5590-5594 (1989)].

It is, therefore, an object of the present invention to provide an aON comprising a nucleotide sequence which is characterized by its ability to hybridize under stringent hybridization conditions to a part of the mRNA sequence of the human progesterone receptor and further characterized by its ability to block the synthesis of the human progesterone receptor or more specifically such an aON which is not longer than 100 nucleotides, preferentially which is 10 to 50 nucleotides long. A furthermore preferred aON is such an aON as mentioned above whereby the part of the mRNA sequence of the human progesterone receptor has the following nucleotide sequence:

5'-GUCAUGACUGAGCUGAAG-3'

or more specifically such an aON comprising the following nucleotide sequence:

5'-CTTCAGCTCAGTCATGAC-3'

or more specifically such an aON which has the following nucleotide sequence:

5'-CTTCAGCTCAGTCATGAC-3'.

A furthermore preferred aON is an aON as mentioned above whereby the part of said mRNA sequence of the human progesterone receptor has the nucleotide sequence which corresponds to nucleotides 641-701 of the corresponding cDNA sequence.

It is furthermore an object of the present invention to provide a process for the preparation of such aONs and modifications thereof characterized in that a protected solid support bound oligonucleotide of corresponding nucleotide sequence which has been synthesized by solid phase synthesis is deprotected and cleaved from the solid support under alkaline conditions, whereby NH_3 is preferred to create such alkaline conditions and which aONs are if desired, accordingly modified. Such aONs and modifications thereof whenever prepared according to such a process are also an object of the present invention.

It is furthermore an object of the present invention to provide a pharmaceutical composition containing at least one aON of the invention or modification thereof and a therapeutically acceptable carrier material.

Finally, the use of such aONs or modifications thereof for the preparation of a pharmaceutical composition, especially for termination of pregnancy are also an object of the present invention.

The invention will be better understood on the basis of the following description when considered in connection with the accompanying drawings, wherein:

Figure 1 shows the effect of the aON of Example 1 on the level of expression of the hPR of T47D-(A) and on MCF7 cells (B). Spotted boxes refer to the experiment performed as described in Example 2 in the presence of the aON and open boxes to this experiment without the aON ("control"), whereby "II" refers to such experiments made in the presence of β-estradiol and "I" to such experiments without the presence of β-estradiol. Data shown represent means +/- standard deviation of means indicated by a bar on top of the boxes of a minimum of 5 single determinations with a probability "p" ≤ 0.05 of control (*) and "p" ≤ 0.01 of control (**).

Figure 2 shows the dose dependent effect of the aON of Example 1 on the level of expression of the hPR on T47D cells whereby "control" has

the same significance as given for Figure 1 and the different doses are indicated as concentrations (1.5 µM up to 12 µM) of the aON used.

Figure 3 shows the antagonistic affect of the aON of Example 1 on progesterone-induced estrogen receptor (ER) down regulation. Spotted boxes refer to the experiment performed as described in Example 3 in the presence of the aON, shadow boxes refer to progesterone treatment and open boxes are controls (no adition of aON nor hormone). The data shown represent means +/- standard deviation indicated by a bar on top of the boxes of a minimum of 5 single determinations with a probability "p"≤0.01 of control (**) and "p"≤0.05 of progesterone treatment at same time (Δ) as determined by the one-way ANOVA statistical test using the "Epistat"-program (available from Tracy L. Gustafson, M.D., 1705 Galtis School Road, Round Rock, Texas 78664, USA).

aONs of the present invention can be designed on the basis of the known mRNA sequence of the hPR [Misrahi et al., Biochem. Biophys. Res. Comm. 143, 740-748 (1987)] whereby regions like splicing sites, ribosomal binding sites, sites for the binding of regulatory proteins, parts of the mRNA responsible for the formation of secondary or tertiary structure of the mRNA or polyadenylation sites, if present and specifically the site around the start codon, are preferred. General considerations with respect to the design of oligo-nucleotides and especially regarding the stringency of hybridization conditions can be found in the state of the art, e.g. in Sambrook et al. "Molecular Cloning, A Laboratory Manual, especially Chapter 11, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, Cold Spring Harbor Laboratory Press (1989)". For the purpose of the present invention it is understood that only aONs of a specific length of their nucleotide sequence are considered suitable. The minimal length of such a sequence can be determined by a man skilled in the art according to the generally known considerations regarding sufficient specificity for a given target sequence of interest (see e.g. in Sambrook et al., see above). The maximal length of such a sequence can be determined also by such a person according to considerations known in the art regarding sufficient penetration efficiency through cell membranes [Loke et al., PNAS 86, 3474-3478 (1989); Yakubov et al., PNAS 86, 6454-6458 (1989)].

Once a specific sequence for an aON of the present invention has been defined according to the criteria mentioned above, such aON can be synthesized in liquid or solid phase by methods known in the art and described for example by Narang [Tetrahedron 39, 3 (1983)], Itakura et al. [Ann. Rev. Biochem. 53, 323 (1984)] or in Chimia 41, 302 (1984), Science 230, 281 (1985) or in "Oligonucleotide Synthesis: A Practical Approach", IRL Press, Oxford, UK, M.J. Gait, Ed. (1984).

If it is desirable, such aONs can be purified by methods known in the art, e.g. by polyacrylamide gel electrophoresis under denaturing conditions or by reverse phase chromatography on silica gel columns, as described for example in Sambrook et al. (see above).

The aONs of the present invention can be characterized by their ability of blocking the synthesis of the hPR, e.g. by blocking the translation of the mRNA of the hPR, e.g. via hybridization of an aON of the present invention to the mRNA. Blocking of translation can be determined by any assay known in the art directed to translation of mRNA, e.g. in vitro translation of mRNA in wheat germ extracts or reticulocyte lysates (see e.g. Sambrook et al.) or by injection into frog oocytes [see e.g. Kawasaki, E.S., Nucleic Acid Res. 13, 4991 (1985)]. In such cases, mRNA has to be isolated from specific tissues known to produce high amounts of hPR by methods known in the art (see e.g. Sambrook et al.), translated in these assays and the blocking of translation measured by a reduced synthesis rate of the translation product. Such measurement can be performed according to any method used for the detection of a specific protein especially a method used for the detection of the binding of a ligand to a receptor, whereby enzyme immun-linked assays as e.g. specifically described in Example 2 are preferred.

Blocking of hPR synthesis by aONs of the present invention, e.g. in a dose dependent manner can be determined also in cultures of cells which are known for their capability of synthesizing hPR at high concentrations, e.g. cultures of cell lines "T47D" (ATCC No. HTB 133) or "MCF7" (ATCC No. HTN 22) and as specifically described, e.g. in Example 2. More significantly blocking of hPR synthesis can be determined by using the same cell culture assays however, under conditions wherein the hPR synthesis is induced by estrogen [see e.g. Horwitz and McGuire, J. Biol. Chem. 253, 2223 (1978); Eckert and Katzenellenbogen, Cancer Res. 42, 139 (1982); Kassis et al., Endocrinology 114, 1558 (1984) and Nardulli et al., Endocrinology 122, 935 (1988)] and as described

e.g. in detail also in Example 2. For the purpose of the present invention cell culture assays are preferred for the determination of the blocking of hPR synthesis since such assays are much closer to physiological conditions as in vitro translation assays mentioned above.

Furthermore aONs of the present invention can be characterized in in vivo models, e.g. by injection into the ventromedial hypothalamus of estrogen-primed female rats and analyzing the resulting change in sexual behaviour and thereby demonstrating a counter action of the physiological action of progesterone.

It is well known in the art that aONs of the present invention can be modified in order to improve their desired properties [for a general review also with respect to general considerations regarding the design of antisense oligonucleotides for the purpose of blocking the synthesis of a specific protein see Uhlmann, E. and Peymann, A., Chem. Reviews 90, 543-584 (1990)]. For example such aONs can be chemically modified at the phosphodiesterbond and/or at the 3'-terminal ends in order to render them more stable with respect to DNAse breakdown or to improve their penetration- or hybridization properties as described for example in the European Patent Application, Publ. No. 386563. Furthermore aONs can be covalently linked to a lipid as described for example in the international patent application with the publication number WO/9010448. In such a manner, DNA can be more efficiently transported across cell membranes and cleaved, e.g. by membrane-bound intracellular cytoplasmic enzymes, to release the active aON.

In order to achieve a higher rate of uptake of aONs into a cell, aONs can also be phosphorylated or linked to cholesterine or derivatives of cholesterol at the 3'end [Letsinger et al., PNAS, 86, 6553-6556 (1989)].

When aONs are modified in order to render them more stable against DNAse breakdown or for better uptake into cells as mentioned above, care has to be taken that such modified aONs still hybridize well to their specific target mRNA and that RNA bound in these complexes can be easily degraded by RNAse H.

Furthermore, aONs of the present invention can be modified in order to ensure the specific delivery of the drug to progesterone receptor synthesizing tissues and, therefore, limiting the dispersion of the molecule administered.

The aONs of the present invention may be administered in pharmaceutically acceptable forms, especially in a form suitable for oral application. Dosage forms and dose rates may parallel those currently being used in clinical applications of known compounds of comparable structure. Pharmaceutical compositions may contain at least one aON of the present invention in association with pharmaceutically acceptable solid or liquid carrier materials. Any conventionally used carrier material can be utilized. Furthermore, the pharmaceutical compositions may contain other pharmaceutically active agents and be prepared by methods known in the art.

Also, the use of aONs of the present invention and their modifications for the preparation of such pharmaceutical compositions and for a diagnostic or medical purposes, e.g. for the termination of a pregnancy or as an anticancer agent [see e.g. Clarke and Sutherland, Endocrine Reviews 11, 266-301 (1990)] are also an object of the present invention.

Furthermore, aONs of the present invention can be used when fixed onto a solid support according to methods known in the art for the isolation or the oligonucleotides itself for the detection of hPR-mRNA. For such diagnostic purposes aONs of the present invention can be labeled with a signalling moiety, e.g. a radioactive isotope, an enzyme, or a fluorescent compound according to methods known in the art.

Having now generally described the invention, the same will become better understood by reference to the specific examples which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified.

Examples

Example 1

Synthesis of an aON

The aON with the following nucleotide sequence was synthesized on a DNA synthesizer (Milligen/Biosearc 7500) according to the instructions of the manufacturer:

5'-CTTCAGCTCAGTCATGAC-3'

Example 2

In vitro activity of an aON

At day one MCF7- or T47D-cells were cultured in RPMI "1640" medium [Pool Bioanalysis Italiana, Milano, Italy] containing 10% fetal calf serum (FCS) at a density of 2-5x10^5 cells/well. At day 3 the medium was replaced by an RPMI 1640 medium without phenol red [ICN Biomedical Ltd., Bucks, GB, Catalog No. 07312854500] and 10% dextran charcoal-stripped FCS with and without 10^{-8} M β-estradiol and 20 µg of an aON as prepared in Example 1 were added. At day 4 additional 10 µg per well of the same aON were added. At day 5 the cells were washed with phosphate-buffered saline (PBS) harvested with trypsin, centrifuged 10 min., at 350xg at room temperature. The cellular pellets were resuspend in 10 mM Tris/1,5 mM EDTA/5 mM Na_2MoO_4/0.4 M KCl, homogenated and centrifuged 1 h at 100.000xg. In the supernatant the amount of hPR was determined by the enzyme immune linked assay of Abbott (Abbott Laboratories, North Chicago, IL, USA) according to the instructions of the manufacturer and expressed as fmoles/mg of cytosolic proteins. Amount of protein was determined according to Bradford et al. [Analyt. Biochem. 72, 248-259 (1976)]. For the determination of the activity of the aON in a dose dependent manner T47D cells were plated in a Costar (Europe Ltd., Badhoeverdorp, Netherlands) 24 wells plate (300.000 cells/well). The cells were grown at semi-confluency in RPMI medium including phenol red, 10% FCS and 10^{-8} M β-estradiol. The aON was added twice: the first time at the concentration shown in Figure 2 and 24 hours later at half of this concentration.

Example 3

Effect of aON on Progesterone-induced ER-down regulation

At day one T47D-cells were cultured in RPMI-1640 medium without phenol red and 10% dextran charcoal stripped FCS and 20 µg of an aON as prepared in Example 1 were added. At day 2 additional 10 µg of the same aON were added. At day 3 the medium was replaced and 10^{-8}M progesterone and 20 µg of the same aON were added. After 8 and 16 hours the cells were killed and the receptor extracts were prepared as in Example 2. In these receptor extracts the amount of ER was determined with an enzyme immune linked assay of Abbott (s.a.) as described in Example 2.

CLAIMS

1. An antisense oligonucleotide comprising a nucleotide sequence which is characterized by its ability to hybridize under stringent hybridization conditions to a part of the mRNA sequence of the human progesterone receptor and further characterized by its ability to block the synthesis of the human progesterone receptor.

2.An antisense oligonucleotide according to claim 1 which is not longer than 100 nucleotides long.

3. An antisense oligonucleotide according to claim 2 which is 10 to 50 oligonucleotides long.

4. An antisense oligonucleotide as claimed in any one of claims 1 to 3 whereby the part of the mRNA sequence of the human progesterone receptor has the following nucleotide sequence:

5'-GUCAUGACUGAGCUGAAG-3'

5. An antisense oligonucleotide as claimed in any one of claims 1 to 4 comprising the following nucleotide sequence:

5'-CTTCAGCTCAGTCATGAC-3'

6. An antisense oligonucleotide as claimed in claim 5 which has the following nucleotide sequence:

5'-CTTCAGCTCAGTCATGAC-3'

7. An antisense oligonucleotide as claimed in claim 1 which has the following nucleotide sequence:

5'-CGGACCGGCTCATGAGC-3'

8. A modification of an antisense oligonucleotide as claimed in any one of claims 1-7.

9. A process of the preparation of an antisense oligonucleotide as claimed in any one of claims 1 to 8,

characterized in that a protected solid support bound oligonucleotide of corresponding nucleotide sequence, which has been synthesized by solid phase synthesis, is deprotected, cleaved from the solid support under alkaline conditions and if desired, modified accordingly.

10. A pharmaceutical composition containing at least one antisense oligonucleotide as claimed in any one of claims 1 to 8 and a therapeutically acceptable carrier material.

11. The use of an antisense oligonucleotide as claimed in any one of claims 1 to 8 for the preparation of a pharmaceutical composition.

12. The use of an antisense oligonucleotide probe as claimed in any one of claims 1 to 8 as a therapeutic agent.

Fig.1

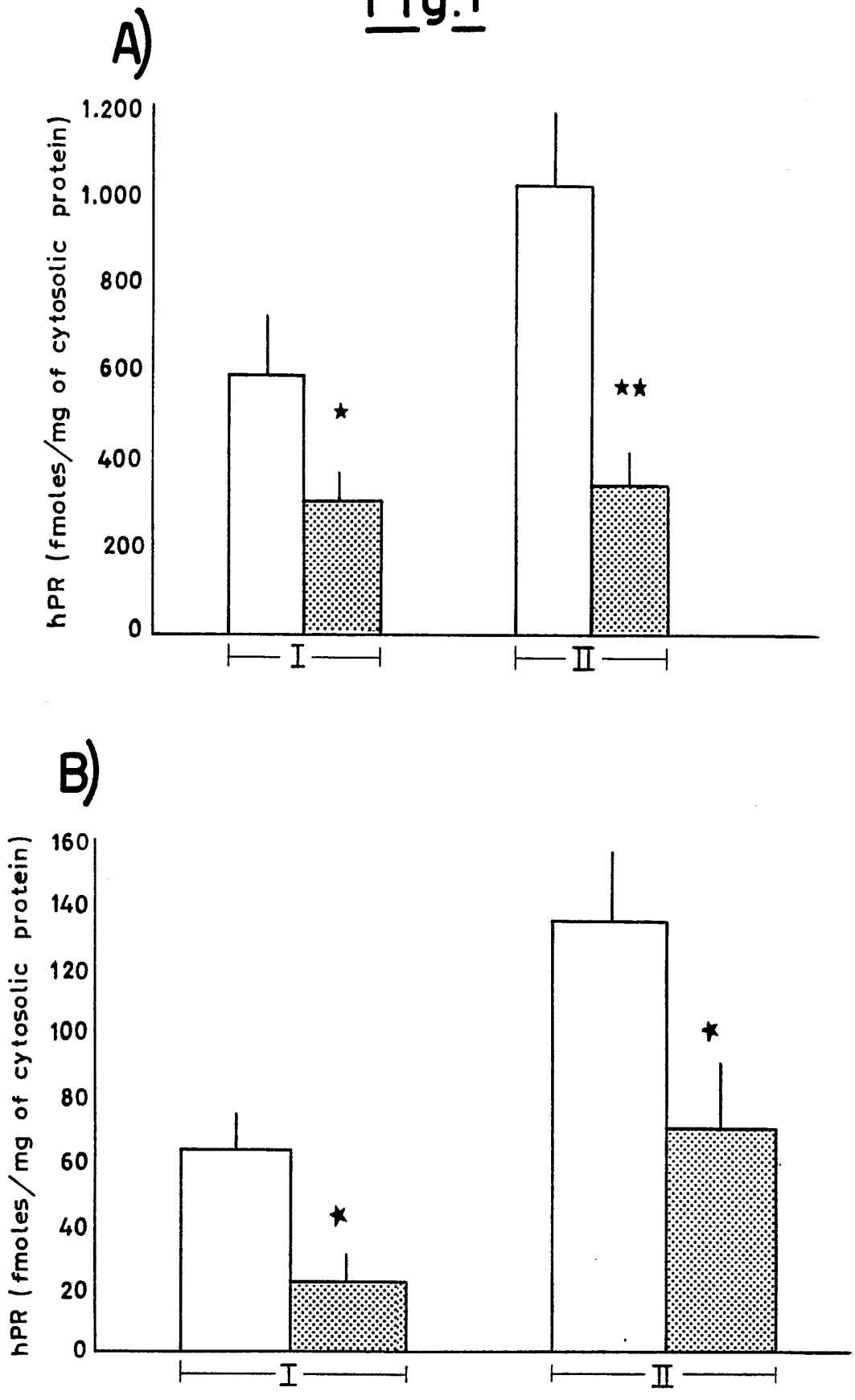

A)
hPR (fmoles/mg of cytosolic protein)
1.200
1.000
800
600
400
200
0
*
**
I
II
B)
hPR (fmoles/mg of cytosolic protein)
160
140
120
100
80
60
40
20
0
*
*
I
II

Fig.2

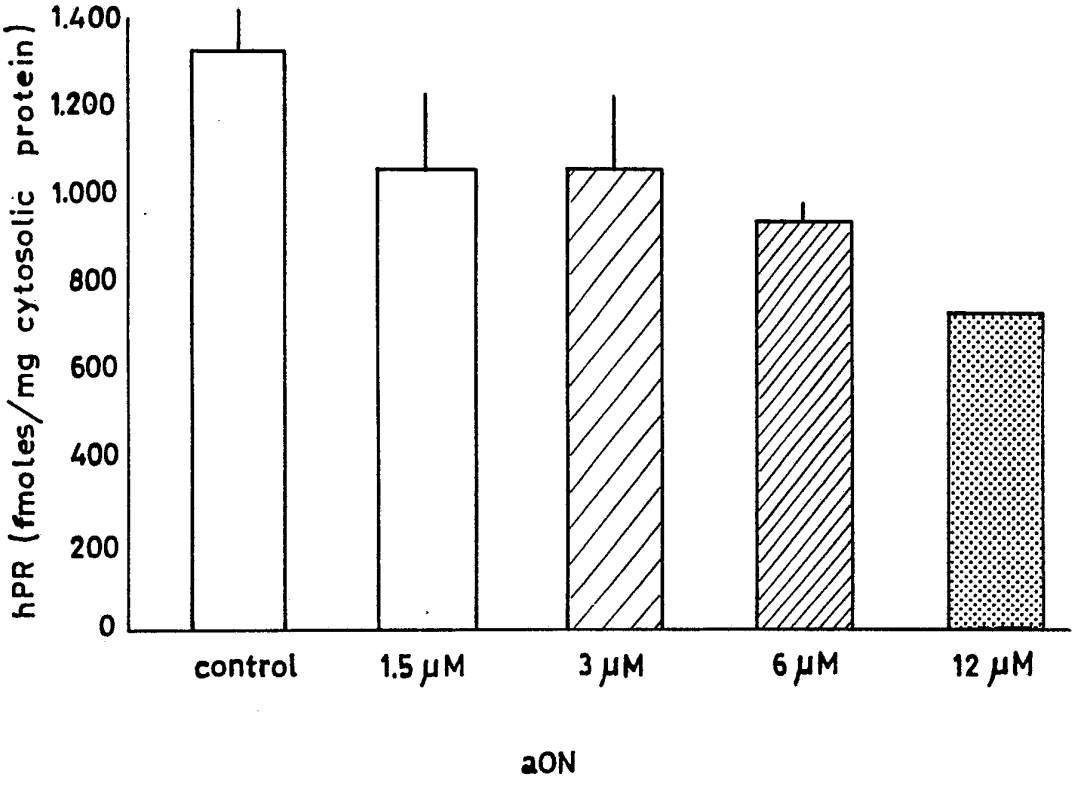
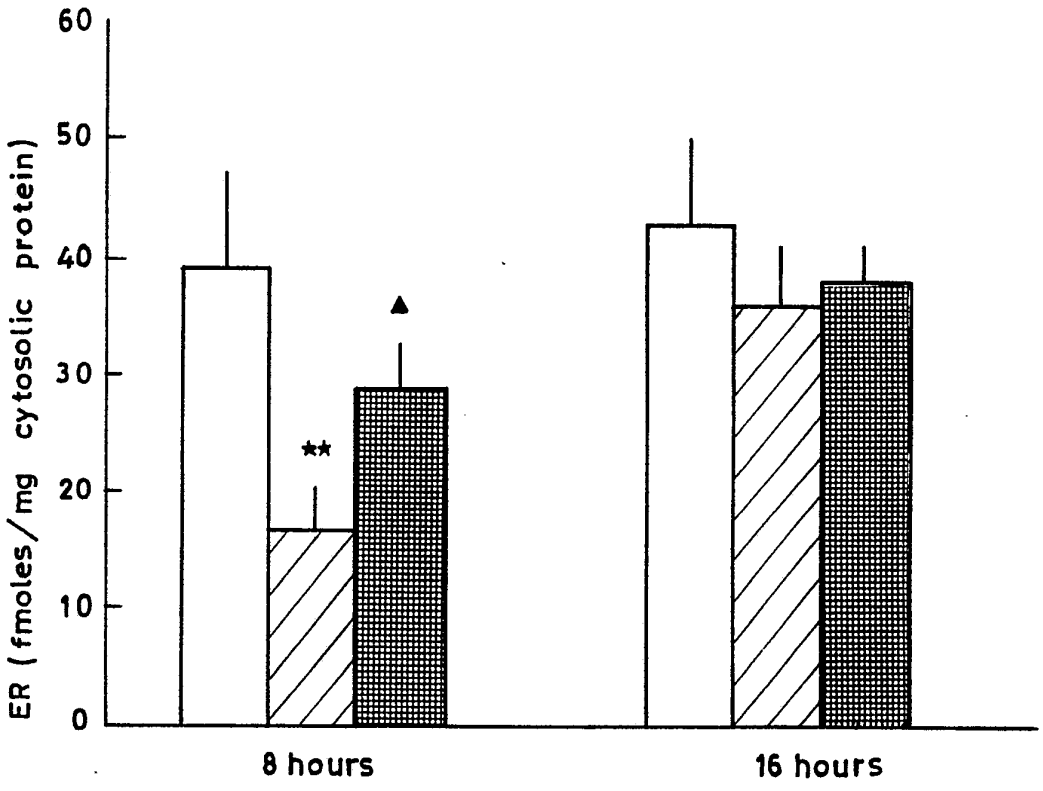

hPR (fmoles/mg cytosolic protein)
1.400
1.200
1.000
800
600
400
200
0
control
1.5 µM
3 µM
6 µM
12 µM
aON

Fig.3


60
50
40
30
20
10
0
ER (fmoles/mg cytosolic protein)
**
▲
8 hours
16 hours

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/01745

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 H 21/04

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 H 21/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A2, 0 414 607 (ROUSSEL-UCLAF) 27 February 1991 (27:02.91), see claims. --	1-12
A	EP, A1, 0 386 563 (BAYER AG) 12 September 1990 (12.09.90), see claims. --	8-12
A	PROCEEDINGS ON THE NATIONAL ACADEMY OF SCIENCES, vol. 86, September 1989, USA, R.L. LETSINGER et al. "Cholesteryl-conjugated oligonucleotides", pages 6533-6556, see the whole document. ----	9-12

* Special categories of cited documents: [14]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 October 1992	13 NOV 1992
International Searching Authority	**Signature of Authorized Officer**
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ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif á la demande de brevet international n°

PCT/EP 92/01745 SAE 62744

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This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

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EP A2 414607	27-02-91	AU A1 61190/90	28-02-91
		CA AA 2023899	24-02-91
		CN A 1049668	06-03-91
		EP A3 414607	03-04-91
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		FR B1 2651130	13-12-91
		HU A0 905302	28-02-91
		HU A2 56113	29-07-91
		IL A0 95342	30-06-91
		JP A2 3091485	17-04-91
		PT A 95067	18-04-91
		ZA A 9006675	30-10-91
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