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**D E C I S I O N**  
of 16 December 2003

**Case Number:** T 0166/01 - 3.3.4

**Application Number:** 92921468.2

**Publication Number:** 0607268

**IPC:** C12Q 1/00

**Language of the proceedings:** EN

**Title of invention:**

An in vitro method of evaluating the effects of a substance

**Patentee:**

KARO BIO AB

**Opponent:**

Akzo Nobel N.V.

**Headword:**

In vitro method/KARO BIO AB

**Relevant legal provisions:**

EPC Art. 123(2), 54, 56

**Keyword:**

"Main request - inventive step (no)"  
"First auxiliary request: added subject-matter (yes)"  
"Second auxiliary request - inventive step (no)"

**Decisions cited:**

G 0010/91, T 0305/87

**Catchword:**

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Case Number: T 0166/01 - 3.3.4

**D E C I S I O N**  
of the Technical Board of Appeal 3.3.4  
of 16 December 2003

**Appellant:** Akzo Nobel N.V.  
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**Decision under appeal:** Interlocutory decision of the Opposition  
Division of the European Patent Office posted  
7 December 2000 concerning maintenance of  
European patent No. 0607268 in amended form.

**Composition of the Board:**

**Chairman:** U. Kinkeldey  
**Members:** R. Gramaglia  
R. Moufang

## Summary of Facts and Submissions

- I. European Patent No. 0 607 268 (application No. 92 921 468.2) claiming priority from SE 9102901 of 7 October 1991 (P) was filed on 6 October 1992. The patent was granted on the basis of 13 claims.
- II. A notice of opposition was filed by the opponent, requesting the revocation of the European patent on the grounds of Article 100 (a) EPC. By a decision dated 7 December 2000, the opposition division maintained the patent on the basis of the claims of the main request then on file, of which claims 1 and 8 read as follows:
- "1. An *in vitro* method of evaluating the tissue specific pattern of antagonistic versus agonistic effects of a receptor-binding test substance in which the following steps a)-h) are performed separately on each type of at least two selected types of *human* cells which contain endogenous intra-cellular hormone receptors and which derive from different kinds of tissues:
- a. that a sample of said cells, in a defined hormone-depleted first medium, is distributed into several separate culture containers, such as microtiter wells,
  - b. that the containers a) are incubated in a temperature and humidity controlled chamber for an appropriate time for the establishment of stable cell growth,

- c. that following b), the spent first medium is replaced by a defined hormone-depleted second medium,
- d. that the equally treated containers of b) are divided into four groups,  $d_1$ ) to  $d_4$ )), each comprising at least one container,  $d^1$ ) to  $d^4$ ), respectively, and each container being treated in the subsequent steps,
- e. that to a container:

$d^1$ ) is added said test substance, dissolved in a first solvent, at a known concentration,

$d^2$ ) is added a reference substance, known to be either an antagonist or an agonist, dissolved in a second solvent, at a concentration known to result in a distinct cellular response selected to be analyzed,

$d^3$ ) is added said first solvent and said second solvent,

$d^4$ ) is added said test substance, dissolved in said first solvent, at the same concentration as used for  $d^1$ ), and said reference substance, dissolved in said second solvent, at the same concentration as used for a container  $d^2$ ),

the first solvent and the second solvent being the same or different, and the amount of the first solvent and the amount of the second solvent not exceeding a level known to be harmful to the cells,

- f. that all the containers d<sup>1</sup>) to d<sup>4</sup>) are incubated in a temperature and humidity controlled chamber for a period of time sufficient for the substances to affect the cells to such a degree that a distinct cellular response selected to be analyzed is reached,
- g. that the incubated containers from step f) are all analyzed with regard to the magnitude of the selected cellular response resulting from hormone/receptor interaction, and
- h. that the antagonistic versus agonistic effects of said test substance on said selected type of cells are evaluated from a comparison of the analyzed magnitudes of the selected cellular response obtained for said groups d<sub>1</sub>) to d<sub>4</sub>)),  
  
and the results obtained for each selected type of cells form together the pattern of antagonistic versus agonistic effects of said receptor-binding test substance on said selected different kinds of tissues.

8. A method according to claim 1, wherein the cells of the selected type contain receptors which are members of the group consisting of steroid hormone receptors, thyroid hormone receptors and vitamin D receptors."

Claims 2 to 7 and 9 to 12 were addressed to specific embodiments of the method of claim 1.

III. The appellant (opponent) filed an appeal against the decision of the opposition division. The board issued a

communication pursuant to Article 11(2) of the rules of procedure of the Boards of Appeal expressing its provisional opinion.

IV. Oral proceedings were held on 16 December 2003, during which the respondent maintained as main request the claims found allowable by the opposition division (see supra) and submitted a first and second auxiliary request. Claim 1 of the first auxiliary request differed from claim 1 of the main request by the addition of the wording "having been 2 x DCC treated" before "first medium" (step a) and "second medium" (step b). Claim 1 of the second auxiliary request differed from claim 1 of the main request by the addition of the wording "including 2 x D.C.C. treated foetal calf serum" after "first medium" (steps a) and "second medium" (step b). Furthermore, compared with claim 8 of the main request, the wording "thyroid hormone receptors" was no longer present in claim 8 of the second auxiliary request.

V. The following documents are cited in the present decision:

(D3) Shapira A. et al., Arch. Otolaryngol. Head Neck Surg., Vol. 112, pages 1151-1158 (November 1986);

(D5) Sheen Y.Y. et al., Endocrinology, Vol. 120, No. 3, pages 1140-1151 (1987);

(D27) Poulin R. et al., Breast Cancer Research and Treatment, Vol. 17, pages 197-210 (1990);

- (D29) Jamil A. et al., Journal of Molecular Endocrinology, Vol. 6, pages 215-221 (1991);
- (D32) Berthois Y. et al., Proc. Natl. Acad. Sci. USA, Vol. 83, pages 2496-2500 (1986);
- (D33) Landau S.I. (Editor in Chief) et al 1986, International Dictionary of Medicine and Biology, Vol. II, Publ. John Wiley and Sons New York, pages 1335-1338;
- (D34) Certificate of analysis from HyClone<sup>7</sup>, Logan, Utah (2002);
- (D35) Product Listing of HyClone<sup>7</sup> (1999);
- (D36) Publication from HyClone<sup>7</sup> on their DCC-FCS (2000).

VI. The submissions by the appellant can be summarized as follows:

*Admissibility of documents (D33) to (D36)*

- These documents were introduced for elucidating the meaning of the term "hormone-depleted" in claim 1 of all requests, ie an issue critical for the first instance to arrive at the decision under appeal.

*Main request*

*Sufficiency of disclosure (Article 83 EPC)*

- Interpreting the wording "hormone-depleted" in claim 1 as meaning that "any substance having hormonal activity, regardless of whether the substance occurs naturally in the body had to be removed from the medium" rendered the patent in suit insufficient under Article 83 EPC, as no information was given in the specification as to how to remove all the hormones.

*Novelty (Article 54 EPC)*

- The Examples in the patent in suit (see eg page 5, line 26) provided a clear and unambiguous definition of what the term "hormone-depleted" meant, namely twice treatment of foetal calf serum (FCS) with dextran coated charcoal (2 x DCC). The skilled person would thus understand that hormones (natural molecules having tissue signalling function: see document (D33)) were excluded to the extent that FCS could be hormone-depleted by means of DCC, even if there remained still significant amounts of eg the hormones corticosterone, thyroxine, triiodothyronine and prostaglandin in DCC-treated FCS (see documents (D34) to (D36)).
- With the above definition of the term "hormone-depleted" provided by the patent in suit, document (D3) disclosed all the features of the method of claim 1 at issue since it related to comparing the antagonistic versus agonistic effects of tamoxifen citrate in six human laryngeal squamous cell carcinoma (SCC) cell lines and the human MCF-7 breast cancer cell line, wherein a first and a second medium (both hormone-



depleted by DCC treatment) had been used. The fact that the medium described by document (D3) may have still comprised the "synthetic hormone" phenol red could not make the claimed method novel over document (D3), as the feature that the medium had to be devoid of such synthetic hormonal activity was not in claim 1.

- Documents (D27) and (D29) also disclosed all the features of the method of claim 1 at issue.

*Inventive step (Article 56 EPC)*

- Departing from document (D3) as closest prior art, the alleged difference between the method described in document (D3) and in claim 1 was the presence in the medium of document (D3) of phenol red. In the light of document (D5) (see page 1141, under the heading "Cells and culture conditions", lines 11 to 14), document (D29) (see page 216, "Cell growth") or (D32), the skilled person would have been aware of problems associated with phenol red, and would have used the appropriate medium without it.
- Departing from document (D29) as closest prior art, the difference from the method of claim 1 was that the medium described in document (D29) contained insulin. Faced with the problem of providing a medium giving the minimum possible interference, the skilled person would have looked to the prior art for suitable media for assays with estrogens. Document (D5) (see page 1141, 1-h column, last paragraph) and document (D32) (see

under "Materials and Methods") both taught the use of media in the absence of phenol red and insulin. Moreover document (D27) (see page 200, under "Cell growth experiments" and Figure 2) used phenol red-free medium and taught that insulin interfered with cell growth experiments.

- Departing from documents (D5) or (27) or (D32) as closest prior art, the method described in this document differed from the claimed one in that cells from only one type of tissue were used. The problem to be solved was to find other estrogen-responsive target tissues. This was suggested in document (D5) itself (see page 1150, 1-h column, first full paragraph), and the skilled person would have looked at either document (D3) or (D29), which both used cells from two different tissues, for the solution to the problem.

*First auxiliary request*

*Added subject-matter (Article 123(2) EPC)*

- The introduction of the wording "having been 2 x DCC treated" before "first medium" (steps a) and "second medium" (step b) in claim 1 of the first auxiliary request represented added subject-matter.

*Second auxiliary request*

*Added subject-matter (Article 123 (2) EPC)*

- The introduction of the wording "including 2 x D.C.C. treated foetal calf serum" after "first medium" (steps a) and "second medium" (step b) in

claim 1 of this request represented added subject-matter.

*Inventive step (Article 56 EPC)*

- Annex 2 to the respondent's letter of 5 November 2001 showed that 2 x DCC treatment of FCS did not achieve any further depletion of estradiol compared to 1 x DCC treatment disclosed by the prior art documents.
- In any case, document (D32) encouraged the skilled person to remove any hormonal activity from growth mediums.

VII. The submissions by the respondent can be summarized as follows:

*Admissibility of documents (D33) to (D36)*

- There were no good reasons for introducing into the proceedings these late submissions, for which appellant had to bear all additional costs.

*Main request*

*Sufficiency of disclosure (Article 83 EPC)*

- The objection under Article 83 EPC raised by the appellant should not be considered in this appeal proceedings, as consent to admit this new ground into the proceedings was refused.

*Novelty*

- The wording "hormone-depleted" meant, in relation to the medium for culturing cells, a medium which had been produced or treated so there was a reduction of any substance (be it natural or synthetic) having hormonal activity to a level which was no longer relevant to the performance of the assay.
  
- Document (D3) related to the growth inhibition of laryngeal and breast cancer cell lines by tamoxifen. The media used in this investigation contained phenol red exhibiting estrogen activity (see document (D32)) and was therefore not hormone-depleted. Moreover, the cells were grown on media containing tamoxifen (see page 1153, r-h column: "fed with tamoxifen").
  
- As for document (D27), it related to only one cell line (see page 208, l-h column: "breast cancer cell") and the medium comprised estradiol. Further no step "d<sup>2</sup>" could be recognized (see legend to Figure 2).
  
- The medium according to document (D29) was supplemented with hormones such as insulin rather than being "hormone-depleted".

*Inventive step (Article 56 EPC)*

- The fact that the appellant used so many different combinations of prior art documents addressing a variety of problems to be solved, in an attempt to question the inventive step, was a proof that

claimed subject matter fulfilled the requirements of Article 56 EPC.

- The present invention solved the problem of developing a reliable and very sensitive assay to evaluate the antagonistic versus the agonistic effects of test substances using a defined hormone-depleted first medium and a defined hormone-depleted second medium, the method being performed on at least two types of human cells taken from different kinds of human tissue. The techniques of the prior art involved unreliable "controls", if any (see eg Figure 5 of document (D29), showing that "ICI alone" was lower than the "control" itself).
  
- The skilled person would not have combined document (D27) with document (D3) or (D29), since neither document related to solving the problem of developing a screening method of test substances which was species and tissue specific as in the present invention. Document (D27) did not disclose the use of different tissue cells in the same assay. Document (D3) did not disclose the use of hormone-depleted medium nor did it suggest combining at least two different cell lines to assess antagonistic versus agonistic effects of a test substance. As for document (D29), it prescribed that the growth medium had to be supplemented with hormones rather than to be depleted of hormones.
  
- There was no motivation for a person skilled to combine documents (D27) and (D3) to arrive at the

claimed in vitro method. The appellant provided no valid reasons as to why the skilled worker would have combined the two documents.

- There was nothing in documents (D3) or (D5) on their own or combined with documents (D29) or (D32) which would have led the person skilled to the present invention. The skilled person would have rather not combined said documents, since they all involved unreliable "controls", if any (see supra).

*First auxiliary request*

*Added subject-matter (Article 123(2) EPC)*

- The wording "having been 2 x DCC treated" after "first medium" (steps a) and "second medium" (step b) in claim 1 of this request had a basis on page 10, lines 1 to 3, 10 and 22 of the published application as filed (WO 93/07290).

*Second auxiliary request*

*Added subject-matter (Article 123(2) EPC)*

- The wording "including 2 x D.C.C. treated foetal calf serum" after "first medium" (steps a) and "second medium" (step b) in claim 1 of this request had a basis on page 10, lines 1 to 3, 10 and 22 of the published application as filed (WO 93/07290).

*Inventive step (Article 56 EPC)*

- Twice treatment of foetal calf serum with DCC in the medium used in the claimed method (cf "including 2 x D.C.C. treated foetal calf serum" in claim 1) was a key-feature to achieving a very high sensitivity, unlike the techniques of the prior art, which involved unreliable "controls", if any. Document (D36) taken as expert opinion showed that a single DCC treatment was not sufficient to remove all the hormones from the medium (see page 3, central column: "A single treatment would be expected to have less effect").
  
- According to document (D5) (see page 1143, r-h column, end of first full paragraph) a single treatment of the serum with DCC was sufficient to render the medium "virtually free of estrogens". The skilled person had thus no incentive to subject foetal calf serum in the medium used in the prior art method to a further step of treatment with DCC, and to arrive at the highly sensitive method according to claim 1. No prior art document suggested to do so.

VIII. The appellant (opponent) requested that the decision under appeal be set aside and that the European patent No. 0 607 268 be revoked.

The respondent (patentee) requested as main request that the appeal be dismissed or alternatively, that the decision under appeal be set aside and the patent be maintained on the basis of claims 1 to 12 of the first or second auxiliary requests both filed during the oral proceedings.

## **Reasons for the decision**

1. The appeal is admissible.

### *Admissibility of documents (D33) to (D36)*

2. As far as the admissibility of the new citations in the proceedings is concerned (which were filed with the Statement of Grounds), it is pointed out that these documents are relevant for elucidating the meaning of the term "hormone-depleted" in claim 1 of all requests, since they show the effect of treating foetal calf serum with dextran coated charcoal (1 x DCC or 2 x DCC) on the presence of residual hormones. This was/is an issue relevant for the first instance/the board to arrive at the decision under appeal/present decision.

Moreover, these documents do not form a basis for a new line of attack on the patentability of the claimed in vitro method, but rather support the argumentation presented by the opponent already in its grounds for opposition. These documents have also been relied upon by the respondent himself for supporting the presence of an inventive step of the claims of the second auxiliary request (see paragraph VII supra). Whilst the board recognises that the introduction of new documents after the expiry of the nine month opposition period might in certain cases be objectionable (depending especially upon the degree of relevance and the lateness), in the present appeal proceedings the board decided to admit documents (D33) to (D36) into the appeal proceedings having regard to what is set out above. Moreover, since the respondent also argued in



its own favour on the basis of these new documents (see supra), it would not be equitable that the appellant has to bear any additional cost originating from these late submissions.

*Main request*

*Fresh grounds for opposition*

3. According to the opinion of the Enlarged Board of Appeal in case G 10/91 (OJ EPO 1993, 420), fresh grounds for opposition may be considered in appeal proceedings only with the approval of the patentee (see section 18). In the present case, the respondent did not consent, neither in writing nor during the oral proceedings, to the introduction by the appellant of objections under Article 83 EPC. Therefore, the objection under Article 83 EPC raised by the appellant is not considered in these appeal proceedings.

*Novelty (Article 54 EPC)*

*Introduction*

4. Claim 1 is addressed to an in vitro method for evaluating a tissue specific pattern of agonistic versus antagonistic effects of a test substance compared to a reference substance known to have an antagonist/agonist effect, on at least two types of human cells which contain intra-cellular hormone receptors. The assay comprises two distinct phases of cell cultivation. During the first phase the cells are grown and pre-conditioned in a first hormone-depleted medium (growth medium) (steps (a) and (b)) and during the second phase the cells are incubated in a second hormone-depleted medium in four containers (or groups

of containers)  $d^1$ ) to  $d^4$ ), upon exposure to: ( $d^1$ ) the test substance at a known concentration eg  $C_1$ , ( $d^2$ ) a reference substance at a known concentration eg  $C_2$ , ( $d^3$ ) a control and ( $d^4$ ) the test substance + the reference substance at a concentration  $C_1 + C_2$ . The magnitude of the cellular response is then analysed (step (g)) and the tissue specific pattern of antagonistic versus agonistic effects are obtained from comparison of the magnitudes of the cellular responses (step (h)).

5. This method is illustrated in the patent in suit (see eg page 5, under the heading "pS2"), wherein two experiments are performed using the human breast cancer cell lines MCF7 and ZR-75-1, respectively. The selected cellular response to be analysed is the amount of expressed protein pS2 after 48 h (see page 6, line 11), the expression of which is regulated by the intra-cellular estrogen receptor. Estradiol is used as a reference substance and the effects of tamoxifen is evaluated. In the table on page 6 of the patent in suit, the amount of pS2 secreted into the medium relative to the control ( $d^3$ ) set at 1 (see "no hormone added") is determined for the test substance ( $d^1$ ), ie tamoxifen alone (see "Tam (100 nM)"), the reference substance alone ( $d^2$ ), ie estradiol at 10 nM (see " $E_2$  (10 nM)") and the test substance + the reference substance ( $d^4$ ) (see " $E_2$  (10 nM) + Tam (100 nM)"). The obtained tissue specific pattern of antagonistic versus agonistic effects (see page 6, lines 35 to 37) is that pS2 can be induced by 10 nM  $E_2$  in the breast cancer cell lines MCF7 and ZR-75-1. It is further concluded that  $10^{-7}$  M tamoxifen functions as agonist in both the cell lines MCF7 and ZR-75-1 in the absence of  $E_2$  and that  $10^{-7}$  M

tamoxifen functions as an antagonist in the presence of  $10^{-8}$  M (ie 10 nM)  $E_2$ .

*Documents (D3), (D27) and (D29)*

6. These documents are argued by the appellant to be novelty-destroying for the subject matter of claim 1.
  
7. **Document (3)** describes a series of in vitro investigations on the effect of tamoxifen on two cancer cells lines (MCF-7 and UM-SCC). The only relevant experiment is that disclosed in Figure 3 on page 1154 of this document, relating to the "prevention of tamoxifen-induced growth inhibition by estradiol". In brief, the MCF-7 and UM-SCC cells are plated in D5 medium (ie complete Eagle essential medium supplemented with 15% fetal bovine serum (FBS) treated with dextran-coated charcoal (DCC) to remove unconjugated steroid hormones; see under "Materials and Methods" on page 1152) and allowed to reach logarithmic growth phase. From day 4, cells are fed daily with (i) the control medium D5 with or without 0.1% alcohol (drug solvent); (ii) 5  $\mu\text{mol/L}$  tamoxifen citrate and (iii) 5  $\mu\text{mol/L}$  tamoxifen citrate + 0.5  $\mu\text{mol/L}$  or 0.1  $\mu\text{mol/L}$  estradiol at two tamoxifen: estradiol ratios (1:10 or 1:50). The results are commented on in the paragraph bridging pages 1156 and 1157: "When both 5  $\mu\text{mol/L}$  tamoxifen citrate and estradiol (at 1/10 or 1/50 the tamoxifen concentration) were added to logarithmically growing cultures, the growth-inhibitory effect of tamoxifen on MCF-7 was partially blocked so that progressive growth was observed. Under the same conditions, only a slight increase in cell number, over

that in tamoxifen-treated cultures was obtained with UM-SCC-5."

8. The board, however, observes that the experiment illustrated in Figure 3 lacks the further test prescribed by claim 1 (e) at issue, wherein the estradiol reference substance ( $d^2$ ) is used alone at the same concentration as in test ( $d^4$ ), in the present case at 0.1 or 0.5  $\mu\text{mol/L}$  (ie 100 or 500 nM), a test which the authors of document (D3) did not even conceive. It follows that the skilled person is **not** taught whether the observed "progressive growth" (supra) is to be ascribed to the 5  $\mu\text{mol/L}$  tamoxifen citrate or to the estradiol 0.1 or 0.5  $\mu\text{mol/L}$  (ie 100 or 500 nM). Hence, the skilled person is not able to derive from the "progressive growth" shown in Figure 3 any conclusion (cf step h of claim 1) as to the agonist/antagonist behaviour of 5  $\mu\text{mol/L}$  tamoxifen citrate in the presence of estradiol 0.1 or 0.5  $\mu\text{mol/L}$  (ie 100 or 500 nM).
  
9. It is true that in a further experiment (see Figure 5 and page 1154, r-h column, first full paragraph), the effect of estradiol on MCF-7 and UM-SCC cells at concentrations between 1 and 500 nmol/L was examined, however, this experiment was done in a different and not relevant context of the "tamoxifen-inhibited cultures", not in an attempt to elucidate the agonist/antagonist behaviour of 5  $\mu\text{mol/L}$  tamoxifen citrate in the presence of estradiol 0.1 or 0.5  $\mu\text{mol/L}$  (ie 100 or 500 nM). Thus, although the combination of the experiment illustrated in Figure 3 with **some** elements of the experiment of Figure 5 of document (D3) may theoretically yield complete step e of claim 1 at issue, these "scattered elements" are not disclosed as

a **specific combination**, contrary to the requirements set out in eg decision T 305/87 (OJ EPO 1991, 429) that a specific combination has to be pointed out by a prior art document for it to be novelty-destroying. In conclusion, document (D3) does not destroy novelty of the method of claim 1.

10. In the experiment disclosed in Figure 2 of **document (D27)** (see page 201), synthetic progestins CMA, CPA, MPA, MGA, NRE and NRG were added to the medium in the presence or absence of 1 nM estradiol ( $E_2$ ), 500 ng/ml insulin (INS) or both hormones to investigate growth stimulation of ZR-75-1 cells. However, this experiment lacks the further test prescribed by claim 1 (e) at issue, wherein the estradiol reference substance ( $d^2$ ) is used alone at a concentration of 1 nM. In Figure 3 on page 202 of this document, the growth medium is both estrogen- and insulin-free. Growth stimulation of ZR-75-1 cells is also measured in this medium upon addition of the estrogen NRE (norethindrone) or NRG (norgestrel) and the antiestrogen EM-139 (see legend to Figure 3 and page 202, r-h column, under the heading "ER activity"). However, this experiment lacks the further test prescribed by claim 1 (e) at issue, wherein the test substance ( $d^1$ ) is used alone at a concentration, in this specific example, of 300 nM. The control ( $d^3$ ) is also missing. The skilled person is thus not in a position to derive from the growth responses shown in Figure 3 or the ER activities shown in Figure 3 of this document any conclusion (see step h of claim 1) as to the agonist/antagonist behaviour of these synthetic progestins in the presence of the reference substance. This conclusion also applies to the experiment of Figure 4 of document (D27), which is

thus not prejudicial to the novelty of the method of present claim 1.

11. **Document (D29)** describes an in vitro experiment wherein the induction of the progesterone receptor (PR) is measured in the endometrial human cancer Ishikawa cell line and in the human breast cancer ZR-75-1 cell line (ie on "at least two selected types of cells" according to present claim 1) after a 6 day-treatment of the cells preincubated during 6 day with, inter alia, estradiol alone, antagonist ICI 164,384 (hereafter ICI), a combination of both, or nothing. Figure 4 (a) illustrates a diagram of the PR for the Ishikawa cell line versus the added estradiol (OE 1 nM), ICI 1 $\mu$ M, OE + ICI and the control. Figure 5 relates to a diagram of the PR for ZR-75-1 cancer cell line treated in the same manner as the Ishikawa. On page 217, under the heading "Effects of hormone treatment on PR concentration", the authors of document (D29) conclude (cf step h of claim 1) that OE alone stimulates PR in both cells, ICI alone increases PR over control in Ishikawa but decreases it in ZR-75-1 and ICI antagonizes PR induction by OE in both cells.
  
12. However, one difference between the in vitro method of claim 1 at issue and that described in document (D29) lies in the fact that in the latter the cells are preincubated in a first medium "DMEM F12 ITS", which includes 10 nM estradiol and 6.25  $\mu$ g/ml insulin (see page 216, l-h column), contrary to the requirement of step a of claim 1, according to which the first medium has to be "hormone-depleted".

13. In conclusion, no prior art document discloses all the features a to h of the in vitro method of claim 1. Therefore the subject-matter of claim 1 and dependent claims 2 to 12 satisfies the requirements of Article 54 EPC.

*Inventive step (Article 56 EPC)*

*Closest prior art*

14. In order to question the inventive step, the appellant uses a plethora of different combinations of prior art documents addressing a variety of problems to be solved in the light of documents (D3), (D5), (D27), (D29) and (D32) taken each as the closest prior art. The board disagrees to this approach, as explained in the following paragraph.
15. As stated above in point 12, one difference between the in vitro method of claim 1 at issue and that described in document (D29) lies in the preincubation medium which is not "hormone-depleted", contrary to the requirement of step a of claim 1. The analysis of documents (D3) and (D27) made under sections 7 to 10 supra in the context of the novelty issue shows that the teaching of these documents is more remote (and less relevant) than that of document (D29). The same conclusion applies to documents (D5) and (D32). Although both deal with investigating the behaviour of tamoxifen (tam), tam + estradiol (E<sub>2</sub>) and E<sub>2</sub> (see Figure 5 and Figure 3, respectively) according to step h of claim 1 at issue, the experiments are carried out upon only one cell line (MCF-7) **and** the incubation medium is not hormone-depleted, as the paragraph headed "Cells and culture conditions" on page 1141, 1-h column

of document (D5) mentions insulin and hydrocortisone. Page 2497, r-h column, lines 3 to 4 of document (D32) states "Then the medium was changed to phenol red- and insulin-free MEM". This suggests that the previous incubation medium comprised insulin. In conclusion, document (D29) must represent the starting point for any problem-solution approach since it comes closer to the claimed subject-matter than any other prior art document.

*Problem to be solved*

16. Departing from document (D29) as closest prior art, the problem to be solved is the provision of an improved assay to evaluate the antagonistic versus the agonistic effects of test substances.

In the board's judgement, the skilled person was aware of the fact that cells involved in such assay had to be in an unstimulated state and that the incubation medium had to cause the minimum possible hormonal interference, a source of which was both the serum and the medium itself (see document (D32), page 2496, l-h column, second paragraph). On the one hand, document (D5) (see page 1141, l-h column, last full paragraph), prescribed that phenol-red had to be removed from the incubation medium owing to its estrogenic properties (ibidem, page 1143, r-h column, lines 7 to 8). On the other hand it was known that insulin interfered with cell growth experiments (see document (D27), page 201, r-h column, last paragraph). These two documents, thus, taught the skilled reader that hormone-depleted incubation was desirable and consequently it was obvious for the skilled person to arrive at the claimed



in vitro method involving a defined hormone-depleted incubation first medium. Therefore, the respondent's main request is not allowable under the provision of Article 56 EPC.

*First Auxiliary Request*

*Added subject-matter (Article 123 (2) EPC)*

17. Claim 1 of this request differs from claim 1 of the main request by the addition of the wording "having been 2 x DCC treated" before "first medium" (steps a) and "second medium" (step b). In the respondent's view these amendments have a basis on page 10, lines 1 to 3, 10 and 22 of the published application as filed (WO 93/07290).

In the board's view, however, it cannot be derived from the passages pointed out by the respondent that it is the first medium ("A") and the second medium ("B") which are "2 x DCC treated". It is rather the 10% (in medium "A") or 1% (in medium "B") fetal calf serum (FCS) which undergoes such treatment, not the first or second medium as a whole. Therefore, the respondent's first auxiliary request is not allowable under the provision of Article 123(2) EPC.

*Second auxiliary request*

*Added subject-matter (Article 123(2) EPC)*

18. Claim 1 of the second auxiliary request differs from claim 1 of the main request by the addition of the wording "including 2 x D.C.C. treated foetal calf serum" after "first medium" (steps a) and "second medium" (step b). These amendments have a basis on

page 10, lines 1 to 3, 10 and 22 of the published application as filed (WO 93/07290). Moreover, this information that the first and second medium should include 2 x DCC treated foetal calf serum (FCS) belongs to the **general** part of the description relating to the mediums to be used in all the successive experiments.

*Inventive step (Article 56 EPC)*

19. Again the board considers document (D29) as closest prior art, and departing from it the problem to be solved is the provision of an improved assay to evaluate the antagonistic versus the agonistic effects of test substances.

The question to be answered is whether the twice-treatment of foetal calf serum with DCC in the mediums used in the claimed method (cf "including 2 x D.C.C. treated foetal calf serum"), a measure which the respondent views as a key-feature in order to achieve very high sensitivity, is obvious or not.

20. According to document (D5) (see page 1143, r-h column, end of first full paragraph) a single treatment of the serum with DCC was held sufficient to render the medium "virtually free of estrogens". However, once this passage is balanced with the statement in document (D32) (see page 2496, l-h column, second paragraph) that in order to eliminate sources of estrogens from sera, "**considerable** efforts ... have been applied toward the development of serum-free media" (emphasis by the board), it would appear that 1 x DCC-treated foetal calf serum (be it "virtually free of estrogen" or otherwise) was still not the maximum which the skilled

person could aim at in the field of hormone-free mediums. There was thus still a strong incentive to take measures suited to eliminating any residual hormone activity from the serum/medium. One such measure, in the board's view, was obviously to repeat DCC treatment, thus further depleting serum from residual unconjugated steroid hormones which remained adsorbed to DCC (see document (D3), page 1152, under the heading "Dextran-Coated Charcoal Treatment of FBS").

In view of the foregoing, it was obvious for the skilled person to arrive at the claimed in vitro method involving a first and second mediums "including 2 x D.C.C. treated foetal calf serum". Therefore, the respondent's second auxiliary request is also not allowable under the provision of Article 56 EPC.

## **Order**

### **For these reasons it is decided that:**

1. The decision under appeal is set aside.
2. The patent is revoked.

The Registrar:

The Chairwoman:

A. Wallrodt

U. Kinkeldey