Placenta 32 (2011) 531–534

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Comparative analysis of binding affinities to epidermal growth factor receptor of monoclonal antibodies nimotuzumab and cetuximab using different experimental animal models

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ARTICLE INFO

Article history:
Accepted 7 April 2011

Keywords:
Binding assay
Cetuximab
EGF receptor
Nimotuzumab
Placenta

ABSTRACT

Although pharmaco/toxicological studies have always been conducted in pharmacologically relevant species in which the test material is pharmacologically active, the very specificity of many biopharmaceuticals could present challenges in the identification of a relevant species for pharmaco/toxicological studies. Alternative approaches may improve the predictive value of preclinical assessments of species-specific biopharmaceuticals. This could lead to improved decision-making, reduce the number of experimental animals by eliminating non-relevant studies, and decrease the time and cost involved in the drug development process. As an alternative to utilizing traditional animal models, this study investigated the activity of human EGF and the anti-EGF receptor monoclonal antibodies nimotuzumab and cetuximab using the placenta microsomal fraction of different experimental animals. Ligand–receptor binding curves were obtained from the different experimental animal models, and binding constants were calculated based on the Scatchard plots. The constants for human and monkey EGF receptor expressed on the placental extract showed a $K_a < 10^{-8}$ M, while rabbits, mice and rats showed a $K_a > 10^{-8}$ M. The K_a values obtained from animal placentas show that *Macaca fascicularis* and *Cercopitecus aethiops* monkeys are relevant species for studying the pharmaco/toxicological properties of nimotuzumab and cetuximab.

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1. Introduction

Regulatory authorities have relied upon *in vivo* non-human testing of pharmaceuticals to predict the behavior of new molecules in humans since the 1950s. Before a drug may be tested in human safety clinical trials, the regulatory authorities require pharmaco and toxicological assays in one rodent and one non-rodent species. *In vivo* toxicity assays are required for an Investigational New Drug application (IND) to the FDA and EMA [37].

According to ICH guideline S6 (1997) [15], a safety evaluation program for biological/biotechnology derived products should be performed in relevant species, that is, one in which the test material is pharmacologically active due to the expression of the receptor.

However, accurate prediction of the human response to novel pharmaceutical compounds is difficult, often unreliable and invariably expensive. While *in vivo* animal studies can account for the complex inter-cellular and inter-tissue effects not observable from in vitro assays, these studies are expensive, laborious, time consuming, and use a large number of animals. In addition, there is considerable concern as to whether, for example, animal studies are sufficiently predictive of human risk because there is no known mechanistic basis for extrapolation from high to low doses [3] and also, if organisms with different physiology and metabolism on several species could be good models of human being.

To address these limitations, a new comparative ligand–receptor binding study using microsomal fraction of placentas was developed, thus providing a higher and more accurate informational content in order to evaluate the pharmaco and toxicologically relevant animal species for EGFR-targeting monoclonal antibody studies.

This method has the potential to improve the selection of animal species before starting the preclinical testing phase. Earlier prioritization will reduce the number of animals needed for toxicological testing, increase throughput in the preclinical phase, decrease the time and cost of preclinical studies and increase the efficacy of clinical trials.

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0143-4004/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.placenta.2011.04.008

Among the most successful new molecular-targeted drugs for the treatment of cancer are the signal transduction inhibitors. These drugs, in general, target specific molecular alterations that are considered to represent key elements for cellular functions [38].

The epidermal growth factor receptor (EGFR) is a tyrosine-kinase receptor of the ErbB family that is abnormally activated in many epithelial tumours. Overexpression of the EGFR has been associated with a higher degree of malignancy and a poorer prognosis in cancer patients [26]. Two examples of such EGFR-targeting monoclonal antibodies include the humanized monoclonal antibody, nimotuzumab [25], that was obtained by transplanting the complementary determining regions of a murine antibody ior egf/r3, murine version of nimotuzumab MAb, to a human framework of IgG_1 immunoglobulin, and the chimeric monoclonal antibody, cetuximab, where the variable regions of inmunoglobulins have murine origin [12]. These drugs have been used in clinical trials for the treatment of solid tumours [32,33].

In this study, the binding affinity constants of the endogenous ligand EGF, nimotuzumab, and cetuximab to the EGF receptor were comparatively analyzed in placentas from 7 different species frequently used in pharmaco/toxicological studies of pharmaceuticals in order to determine the most relevant species for additional pharmacological and toxicological studies.

Placenta was selected because of its high epidermal growth factor receptors (EGFR) expression; the experiments were performed using a placenta microsomal fraction to determine the EGFR binding affinity of the tested antibodies.

Other studies would be recommendable using placenta tissue to determine absorption, distribution of test agent, metabolism and adverse effects considering, as Schneider and Miller [31] described, that the transport processes for macromolecules through the placenta and extraembryonic membranes occur in different sites depending on species, and therefore is very important to evaluate those differences in order to elucidate the pharmaco and toxicological effects.

2. Materials and methods

2.1. Ethics approvals

Ethical approval for the use of human placenta in these experiments was obtained from the Ethical Committee of the Eusebio Hernández Hospital (Havana, Cuba) in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki)*, and the ethical approval for the use of animal placentas was obtained from the Institutional Animal Care and Use Committee of the National Center for Breeding of Laboratory Animals (CENPALAB, Havana, Cuba) in accordance with *EC Directive 86/609/EEC for animal experiments.*

2.2. Samples

Microsomal placental membrane extracts were obtained from 7 experimental animal models (human, *Macaca fascicularis*, *Cercopitecus aethiops*, New Zealand rabbit, OF1 mouse, NMRI mouse and the Sprague Dawley rat). Placental tissues of human and monkeys were collected from normal term pregnancies. For the non-primate species, placental tissues were collected from normal term pregnancies by elective Cesarean section.

Identity with human EGFR is the following: for monkeys 97%, for mice 84% and for rat and rabbit 86% [27].

Monoclonal antibodies (nimotuzumab and T1h) were obtained from Center for Molecular Immunology, Havana, Cuba, and cetuximab, from Merck KGaA and ImClone Systems Incorporated/Bristol-Myers Squibb.

2.3. Placental microsomal membrane extraction

Thirty grams of human and animal placentas were homogenised in cold tris–HCL buffer 10 mM, pH 7.4 for 1 min [24].

The homogenate was centrifuged at 100,000 *g* for 10 min at 0 °C, then supernatant was collected and centrifuged for 1 h and the microsomal fraction was obtained in the pellet. Protein concentration was determined using Lowry's method [23] and the microsomal fraction was stored at −20 °C until used.

Competitive binding studies were performed between ^{125}I-EGF and different ligands: EGF, nimotuzumab, cetuximab, and T1h as negative control (an anti-CD6 monoclonal antibody). The microsomal placental membrane extracts came from a pool of placentas (2–15 for each experiment in order to achieve 30 g of placental tissue).

2.4. Radioiodination of EGF

EGF was radioiodinated by the chloramine T method as described elsewhere [14].

2.5. Competitive binding assay

Crude membrane fractions (600 μg of protein) were incubated with approximately 100,000 cpm of ^{125}I-EGF (200 μC_i/ug) in a series of different concentrations of unlabelled EGF, nimotuzumab, cetuximab or T1h (0; 0.312; 0.625; 1.25; 2.5; 5; 10; 20; 40; 80; 160 and 1000 ng/mL as final concentrations) added at the same time. Total volume was made up to 500 μL with binding Tris–HCl 10 mM buffer, pH 7.4, 10 mM $MgCl_2$, 0.1% BSA. Samples were incubated for 1 h at room temperature and the reaction stopped with cold binding buffer. After centrifugation at 3000 *g* for 30 min, pellet radioactivity was determined in a gamma counter [24]. The specific binding was defined as the difference between the total and the non-specific binding; non-specific binding was defined as the non-displaced binding by non-labelled ligand greater than 100-fold excess relative to the radiolabelled ligand. The amount of free radioligand was considered to be the difference between the total amounts of bound ligand minus the amount of unlabelled ligand.

2.6. Statistical analysis

The mean and standard deviation were calculated for all K_a. Three experiments were performed in triplicate for each substrate (placentas from Sprague Dawley rat, NMRI mouse, OF1 mouse, New Zealand rabbit, *C. aetiops*, *M. fascicularis*, and human) and ligand (hEGF, cetuximab, nimotuzumab) respectively. Statistical evaluation was performed by means of the Kruskall–Wallis test with significance assessed at the $P < 0.05$ level. When $P \leq 0.05$ was reached, Dunn's test was used for determining at what dose point differences existed for pairs among substrates and ligands.

3. Results and discussion

The specific recognition of the EGF and monoclonal antibodies nimotuzumab and cetuximab for the EGF-R expressed on the membrane of placental extracts from different animal models was determined by a competitive binding assay.

Fig. 1 shows the displacement dose–response curves for EGF, cetuximab and nimotuzumab for the recognition of EGF-R expressed on the placental membrane extract of seven different experimental animal models (human, *M. fascicularis*, *C. aethiops*, New Zealand rabbit, OF1 mouse, NMRI mouse and the Sprague Dawley rat).

EGF, cetuximab and nimotuzumab bind to the EGF receptor on human placenta with an affinity constant K_a of $(0.632 \pm 0.062) \times 10^{-8}$ M, $(0.856 \pm 0.058) \times 10^{-8}$ M and $(4.530 \pm 0.010) \times 10^{-8}$ M respectively (Table 1). No binding was observed for the anti-CD6 humanized antibody T1h (Fig. 1).

Fig. 1 also shows the degree of specific binding for EGF, cetuximab and nimotuzumab on the EGF receptor expressed on the placental membrane extract of 6 other animal models. Different degrees of binding affinity were found in the ligand–receptor binding for each species studied. The human species had the greatest affinity, followed by the *M. fascicularis*, *C. aetiops*, New Zealand rabbit, OF1 mouse, NMRI mouse and the Sprague Dawley rat.

It is generally agreed that there is a problem in the ability of the pharmaceutical industry to introduce effective and safe new medicines to the market. Despite ever increasing research and development expenditure, a more thorough understanding of the molecular basis of disease and the introduction and application of new technologies, new drugs continue to fail in terms of their clinical efficacy and/or safety profiles. The assumption that animal models are reasonably predictive of human outcome provides the basis for their widespread use in toxicity testing and biomedical research aimed at developing cures for human disease. Recently, however, several animal toxicology studies have failed to clearly

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