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Monoclonal anti-epidermal growth factor receptor (ior EGF/r3) antibody pharmacokinetic studies on nude mice I: a radio-receptor analysis applied to drug serum quantification

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Abstract

Due to its antagonistic properties upon ligand–epidermal growth factor receptor (EGFr) interaction, the monoclonal antibody anti-EGFr ior EGF/r3 is considered a potential therapeutic agent against several epithelium-derived tumours. This paper affords further analysis of the relevant corporal interaction of this monoclonal antibody in terms of its pharmacodynamic properties, using nude mice, following a single bolus intravenous dose administration. The radio-receptor assay allows quantification of the serum ior EGF/r3 level. The dose selection procedure, according to the Kolmogorov–Smirnov test, suggested using doses of 12.5–16 mg kg^{-1} for pharmacokinetic assessments. The experimental data were best fitted to a bi-exponential function ($r^2 = 0.985$), through the classical two-compartment open modelling approach. The model selection was corroborated by the AKAIKE information criteria, and also the SCHWARTZ and ESTRIP test were used. The estimated pharmacokinetic parameters (e.g. $t_{\frac{1}{2}\beta}$ = 34.65 h, Vc = 2.84 mL, Vss = 4.21 mL and CL = 0.09 mL h^{-1}) bear out the strategies for the evaluation of the therapeutic application of this drug. Finally, the radio-receptor analysis has provided a rationale for the proposed serum monoclonal antibody ior EGF/r3 quantification to characterize its concentration–time course.

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Introduction

The over-expression of epidermal growth factor receptor (EGFr) and other cell surface glycoprotein members of the oncogene *ErbB* family by undifferentiated epithelial tumours brought on the possibility of developing selective anti-EGFr monoclonal antibodies as an alternative tool for cancer therapy. The human EGFr is a membrane-bound glycoprotein which binds some ligands like EGF and TGFα (transforming growth factor alpha). Several important reports have addressed the close relationship between the EGFr/*ErbB*2 heterodimers and malignancy (Garcia-Allan et al 1997; Moyer et al 1997). The murine monoclonal antibody anti-EGFr ior EGF/r3 (IgG_{2a} isotype) recognizes two conformational sequence-sites of the receptor extracellular domain with high binding affinity ($K_d = 10^{-9}$ M) depending upon its blocking action on ligand binding to the EGFr which partially reduces the receptor autophosphorylation and inhibits the receptor (hetero)-dimerization in intact tumour cells, suggesting a cytostatic response by their diminishing effects in the physiological receptor functions. Beside, the ior EGF/r3 immunohistochemical

stained pattern was similar to the pattern of the standard anti-EGF receptor monoclonal antibody from Amersham (Fernández et al 1989).

Nowadays, the monoclonal antibody ior EGF/r3, like other anti-EGFr monoclonal antibodies, is subject to important clinical and pre-clinical studies to assess its possible therapeutic usefulness. Also, these molecules, used as radio-tracers, may be of diagnostic importance for monitoring different epithelial tumours (Mendelsohn 1989; Brady et al 1990; Fan et al 1993; Goldstein et al 1995). According to the Food and Drug Administration of the United States (1999), when considering pre-clinical tests on monoclonal antibodies and many other biological molecules, pharmacokinetic studies should be undertaken in at least two different animal models including a rodent species. For naked monoclonal antibodies directed at human antigens, as opposed to foreign antigens (viral, bacterial, etc.), studies in animal species lacking the target antigen may not be necessary; preference should always be given to using an animal model that shares a cross-reactive or identical target antigen with humans. The development of anti-immunoglobulin antibodies greatly complicates study and interpretation of effects of repeat dosing in animals. Murine antibodies are non-immunogenic in mice but are immunogenic in humans and other species, making it difficult to extrapolate the results of studies in mice to planned repeat-dose administration in humans; but if a humanized monoclonal antibody is further considered, then the studies in rodents with the murine monoclonal antibody may, in this case, be of great value.

Because pharmacokinetic studies may be useful in the interpretation of pre-clinical drug activity and potential toxicity, and in the recommendation of an appropriate dosing regimen, and thereby improve the design of clinical trials, they may provide the initial step in determining the pharmacodynamic end-points. Here, we show the first part of the monoclonal antibody ior EGF/r3 pharmacokinetic studies using the nude mouse as an animal model since we wanted to use a nude mouse bearing tumour model as an in-vivo model of disease state that carries the relevant human antigen or target. We herein define the pharmacokinetic regularity of this monoclonal antibody ior EGF/r3.

Material and Methods

Animals

Thirty male nude mice of NmRI/ nu-nu strain, from the Center of Molecular Immunology (CIM, Havana, Cuba) were used. They were bred and maintained under controlled conditions during the experiment. These immunodepressed mice were 8–10 weeks old and weighed 25 ± 3 g at the beginning of the experiment and were kept 3 in a cage. According to the experimental requirements mice were separated into two groups: drug treated and saline (control) treated. All procedures were performed as approved by the International Animal Care Committees (ARCA No. 012/2000) and in accordance with the European Union Guidelines for animal experimentation.

Monoclonal antibody ior EGF/r3

The lot used, from the Center of Molecular Immunology (CIM, Havana, Cuba), was produced in compliance with standard quality conditions for these kind of formulations. Each vial contained 50 (± 0.2) mg of sterile monoclonal antibody ior EGF/r3 powder in 10 mL of neutral phosphate-buffered saline (PBS) solution.

Kinetics assay

According to the pharmacokinetic characterization study, mice were deprived of food overnight before the experiment, but were allowed free access to water. Mice in the treated group were inoculated with a 16 mg kg^{-1} single dose of the monoclonal antibody ior EGF/r3, by intravenous injection. Mice in the control group were only treated with saline solution (NaCl 0.09 M).

Blood samples were withdrawn by venipuncture from the ocular plexus, at 0 and 30 min and 1, 1.5, 2, 3, 4, 8, 12, 24, 48 and 72 hours following drug administration. Each time represents the average of 3 replicates. Later, serum was obtained from the blood samples. Finally, the serum samples were stored at -20°C for further drug quantification.

Drug quantification assay

The quantification of drug levels in serum was accomplished by a radio-receptor analysis adapted from a previously reported method for determination of picomolar concentrations of EGF in biological fluids (Macías & Pérez 1985). Briefly, a homogenate of human placental tissues, which express high EGF receptor levels, in Tris-buffer (pH 7.4) was ultra-centrifuged (Centrikon T-1170, UK) at 12000 rpm for 10 min at 4°C. The microsomal fraction was used as reaction membrane (100 μg per reaction tube). A purified [I^{125}]-EGF at 0.1 mg mL^{-1}, radio-labelled by the chloramine-T method (efficiency, 80%; specific activity,

200 μCi μg^{-1}) was used as binding ligand (20 μL per reaction tube). The final volume of each reaction tube was adjusted to 500 μL by adding reaction buffer (pH 7.4). The incubation time was approximately 1 h at 27°C, and then the reaction was stopped by centrifugation at 3000 rpm for 30 min. Later, the residual radioactivity in the sediment was measured by Gamma Counter (LKB, Sweden).

The biological sample (50 μL) that contained the displacement agent ior EGF/r3 was quantified through performed displacement curves using equation 1 and the control displacement-curve data from previously known ior EGF/r3 concentrations (0, 1, 2, 5, 10, 20, 50, 100, 200 ηg mL^{-1}).

$$B/B_0 = m \cdot \log C + b \quad (1)$$

where: B/B_0 corresponds to the performed specific binding data point over experimental time, C is the drug concentration, and m and b are empirical parameters depending on the fitting function. This method showed an intra- and inter-assay variation coefficient of 5%, a limit of sensitivity of 0.16 nM (1 ng mL^{-1}), and a correlation coefficient of 0.93. The optimum concentration of work was near to 3.3 nM (20 ng mL^{-1}) (Macías & Pérez 1985).

Pharmacokinetic analysis

The experimental drug serum concentration data corresponding to the individual mice were fitted to a polyexponential equation interpreted as a classical compartment mammilar open model, using the WinNonlin program (WinNonlin Professional, Version 2.1, Pharsight Inc., 1997, NC). Different exponential models were investigated and the most suitable model was chosen according to the AKAIKE information criterion (Akaike 1976), SCHWARTZ test (Schwartz 1978) and the ESTRIP criteria (Brown & Manno 1978). The resulting basic pharmacokinetic parameters were calculated for each animal by standard methods (Gibaldi & Perrier 1982). Likewise, the peripheral drug concentration–time courses in each mouse were conveniently determined by conventional procedures according to the compartmental modelling analysis using previously estimated pharmacokinetic parameters (Gibaldi & Perrier 1982).

Statistical analysis

This analysis was achieved by the Kolmogorov–Smirnov test ($P \leqslant 0.05$) using the statistical program SPSS for Windows Version 6.0 (SPSS Inc. Corp., 1993), and pursued to determine the optimal dose corresponding to the pharmacokinetic study according to the drug quantification method used. All data points were expressed as media and standard deviation, corresponding to 3 mice per time within each studied group.

Results

Table 1 and Figure 1 reflect the linearity (optimal range) and sensitivity parameters of the radio-receptor analysis method when the displacement agent was the monoclonal antibody ior EGF/r3. The displacement curve,

Table 1 The calibration curve's parameters corresponding to the binding data points of the displacement experiment by radio-receptor analysis using the monoclonal antibody ior EGF/r3 as displacement agent ($r^2 = 0.824$).

Parameter	Value±s.e.m.	Confidence interval
Slope	0.165±0.0463	0.278–0.050
Intercept	0.221±0.042	0.117–0.320
$B/B_{0(0.5)}$	0.245	
$B/B_{0(0.9)}$	0.441	

B/B_0 $(_{0.5})$ represent the specific binding obtained with 50% displacement of the membrane-bound radio-labelled EGF by the ior EGF/r3 concentration (linearity parameter). B/B_0 $(_{0.9})$ represents the specific binding obtained with 90% displacement of the membrane-bound radio-labelled EGF by the ior EGF/r3 concentration (sensitivity parameter).

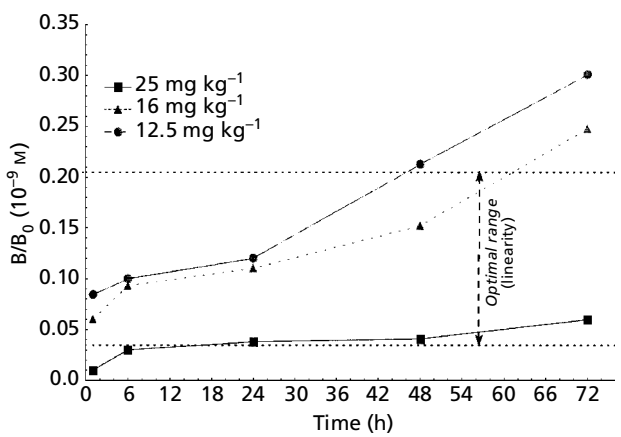

Figure 1 Specific binding (B/B_0) after the EGF tracer displacing effect elicited by the drug (ior EGF/r3) over time, based on mechanisms of step-wise competitive binding equilibrium, using the radio-receptor assay. The experiment was extended to select the better dose, in correspondence with the previously defined linear range, for the subsequent pharmacokinetic studies.

Table 2 The serum monoclonal antibody ior EGF/r3 concentration data points following administration of a single 16 mg kg^{-1} bolus intravenous dose in nude mice, obtained by drug quantification using radio-receptor analysis.

Time (h)	Drug concn (μg mL^{-1})			Average value (±s.e.m.)
	Mouse no. 1	Mouse no. 2	Mouse no. 3	
0.5	138.7	140.1	129.8	136.2 (±5.61)
1	137.2	131.5	134.2	134.3 (±2.86)
1.5	128.2	130.1	124.7	127.7 (±2.78)
2	116.6	120.7	117.4	118.2 (±2.19)
3	112.3	115.3	113.6	113.6 (±1.55)
4	110.9	97.1	99.9	102.6 (±7.26)
8	94.5	91.9	92.0	92.8 (±1.47)
24	78.4	58.7	61.2	66.1 (±10.6)
48	45.2	40.5	47.2	44.3 (±3.44)
72	25.3	29.4	20.8	25.1 (±4.36)

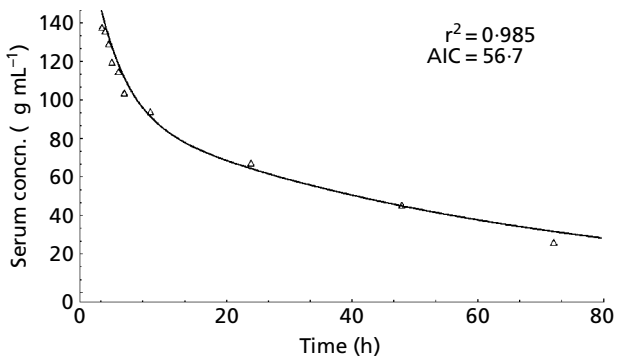

Figure 2 The serum drug disposition over time after the administration of a single intravenous dose of the monoclonal antibody ior EGF/r3 (16 mg kg^{-1}) to nude mice. The classical two-compartment open model was used to fit the experimental data points depicted by open triangles. AIC, AKAIKE information criterion.

Table 3 Selection criteria for the pharmacokinetic models that describe the drug concentration profiles in nude mice after administration of a single bolus intravenous dose of the monoclonal antibody ior EGF/r3.

Criterion	Alternative models	
	Bicompartmental[a]	Monocompartmental
AKAIKE	56.7	71.9
SCHWARTZ	57.3	72.5
ESTRIP	194.5	891.26

[a]The selected model was defined according to the smaller value.

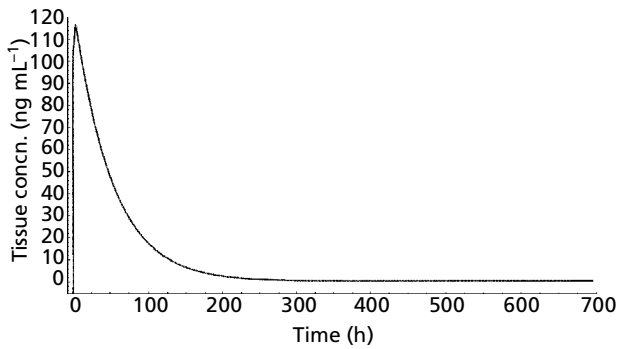

Figure 3 The estimated drug concentration–time course at tissue compartment using a two-compartment open modelling approach. A single 16 mg kg^{-1} intravenous dose of monoclonal antibody ior EGF/r3 was administered to nude mice.

according to the experimental binding data and corresponding to the remainder of the membrane-bound radio-labelled EGF after displacement action of the ior EGF/r3, was fitted to an empirical linear binding function by the least-squares method of regression.

Table 2 lists the performed experimental data points corresponding to the quantified blood drug levels over time. At a glance, it could be appreciated that these values allowed elucidation of a typical time-course for an intravenous bolus dose. Figure 2 depicts the corresponding bi-exponential drug disposition time-course after intravenous administration of 16 mg kg^{-1} of monoclonal antibody ior EGF/r3, which was adequately fitted to a two-compartment open model. The goodness of the fit and the accuracy of the analytical method used were corroborated through the regression coefficient (r^2 = 0.985) and a standard error smaller than 5%, respectively. The estimated AKAIKE information criteria, SCHWARTZ test and ESTRIP F-test criteria, which determine the most valuable and reliable pharmacokinetic model among several alternatives according to its heuristic and pragmatic value, are shown in Table 3.

The drug disposition time-course was quickly completed and at approximately 72 h after treatment the drug serum concentration was negligible. Similarly, the estimated tissue drug concentration peaked at 24 h following treatment and then declined into a downward trajectory, finally returning to baseline at approximately 300 h, as illustrated in Figure 3. Table 4 lists the estimated average values of the pharmacokinetic parameters corresponding to the administration of a single dose of the monoclonal antibody ior EGF/r3 in the nude mice. As we are not aware of any significant non-specific binding of this monoclonal antibody to blood components, the steady-state distribution volume

Table 4 The estimated pharmacokinetic parameters corresponding to the monoclonal antibody ior EGF/r3 following administration of a single 16 mg kg^{-1} bolus intravenous dose in nude mice using a two-compartment open pharmacokinetic model (AKAIKE information criterion = 56.7; r^2 = 0.985).

Parameter	Estimated value
α (h^{-1})	0.82
β (h^{-1})	0.02
A (μg mL^{-1})	109.8
B (μg mL^{-1})	65.9
C_{max} (μg mL^{-1})	175.8
Vc (mL)	2.84
Vp (mL)	1.37
Vss (mL)	4.21
k_{12} (h^{-1})	0.29
k_{21}(h^{-1})	0.52
k_e (h^{-1})	0.03
$t_{\frac{1}{2}}\alpha$ (h)	0.85
$t_{\frac{1}{2}}\beta$ (h)	34.6
MRT (h)	44.76
AUC (μg·h mL^{-1})	5315.6
[a]AUTC (ng·h mL^{-1})	6576.8
CL (mL h^{-1})	0.09
T/B ratio	0.56

[a]AUTC represents the area under the estimated tissue drug concentration–time curve.

showed a relatively poor diffusional drug transfer from blood to the innermost peripheral tissues.

Discussion

Dose selection

To be able to use the radio-receptor analysis method as a reliable and accurate method for measurement of drug concentration in biological matrixes involves making over many relevant issues of this technique. Therefore, we need to seek out a method with which to assess dose selection that allows for quantification of the drug serum level above the cut-off level depending on the sensitivity parameter of the cited method. Accordingly, it is recommended that during drug quantification, some reactants used in the system are readjusted according to our experimental end-point. In this case it may be appropriate to change the displacement agent of the standard displacement curve so as to use the same monoclonal antibody ior EGF/r3 that should be measured in the sampled fluid rather than the previously reported EGF ligand (Macías & Pérez 1985). The substitution of the natural ligand for the monoclonal antibody ior EGF/r3 will pursue the estimation of the empirical parameters of the above mentioned displacement formulae depending on the specific binding data corresponding to this drug at previously known concentrations, which allows further use of this formula to determine the drug concentration in the sampled fluid and thereby increases the validity of the drug amount testing.

Table 5 Statistical parameters according to the performed Kolmogorov–Smirnov test ($P \geqslant 0.05$) to select the most valuable dose for further pharmacokinetic characterization of the monoclonal antibody ior EGF/r3 using radio-receptor assay.

Dose (mg kg^{-1})	Median	Variance	s.e.m.
12.5	0.125[a]	0.092	±0.041
16	0.114[a]	0.070	±0.031
25	0.034	0.018	±0.008

[a]No significant difference with respect to the centroid of the standard displacement curve ($B/B_{0(0.5)}$ = 0.245) corresponding to the linearity range.

A broad dose range should be explored to ensure that differences in distribution and elimination which may occur at extreme doses do not result in non-measurable drug levels. The range of doses selected for study should include at least one dose that is equivalent to, and one dose that is a multiple of, the highest anticipated clinical dose, without necessarily the highest dose tested eliciting any adverse effect. Generally the dose range is best established with a minimum of three doses, thus we evaluated the following single doses: 12.5, 16 and 25 mg kg^{-1} to ensure that the final dose selected for the pharmacokinetic study displayed drug serum levels over time which could be measured within the optimal linearity range, and particularly above the detection limit, of this analytical method. The specific binding data points corresponding to each dose evaluated were analysed according to the median's comparison by the Kolmogorov–Smirnov test. Table 5 represents this statistical analysis, which shown that doses of 12.5 and 16 mg kg^{-1} could be used because both had their medians near to the centroid of the standard displacement curve (i.e. 0.245), whereas the 25 mg kg^{-1} dose was rejected due to its median being outside the linearity range of the method.

Pharmacokinetic analysis

From the analysis of the data shown in Table 3 we are just able to describe a classical distribution–elimination profile, whose kinetic characteristics during the first 6 h

show a rapid distribution phase followed by the corresponding elimination phase. Therefore, we conclude that a pseudo-equilibrium condition was attained by the administered drug before the first 12 h had elapsed, suggesting a differential drug distribution behaviour, probably toward the highest blood-perfused tissues in the central cavity, depending upon the drug polarity, the tissue vascularity and the drug transport mechanisms.

The results listed in Table 5 supported our idea about a systemic drug disposition pattern which reflects a predominant drug residence time into a central compartment rather than a temporary transit time towards a peripheral compartment. Because the beta (elimination) phase accounted for at least 80% of the area under the serum concentration–time curve we realize that, from a pragmatic point of view, the clinical testing schemes and particularly the drug dosing design should be oriented, to the greatest extent feasible, according to the beta half-life parameter. This parameter is related to the drug accumulation pattern and subsequently to its biological action. The alpha half-life parameter is smaller due to the very quick distribution process as opposed to the very slow elimination one (α (0.82) $\gg$ β (0.02)). The alpha phase in the nude mouse would be referred to as a drug distribution process by capillary mechanism of transport rather than by the typical diffusion mechanisms (Schlom et al 1990). Therefore, the drug residence time in the body should be predominantly at highly blood-perfused tissues where the systemic drug clearance is concentrated.

Granting that this animal model lacks the target human antigen, the drug disposition must be determined by the drug capillary movement among accessible tissues, and particularly by the hepatic uptake, which is considered as its primary metabolic site, and the renal filtration. Perhaps the murine nature of the drug may be responsible for its relatively large residence time in the body, and its relatively reduced systemic clearance (CL = 0.9 mL h^{-1}). The estimated tissue/blood ratio, smaller than unity, suggests that this monoclonal antibody shows a disposition profile pointed towards a central compartment as already mentioned. Nonetheless, we could possibly expect a markedly pharmacological target-mediated drug disposition if the animal model was xenografts using a human tumour. It is due to the EGFr (target antigen) over-expressed on tumour cells being the only specific binding site for the drug. In further pharmacokinetic studies, using other animal species, we need to evaluate the correspondence between both pharmacokinetic regularities depending on several dosing schemes. Also, a non-compartmental analysis should be considered.

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