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Functional responses and *in vivo* anti-tumour activity of h7C10: A humanised monoclonal antibody with neutralising activity against the insulin-like growth factor-1 (IGF-1) receptor and insulin/IGF-1 hybrid receptors

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ABSTRACT

A novel humanised monoclonal antibody (Mab, h7C10) was raised against the human insulin-like growth factor-1 receptor (IGF-1R); it exhibited potent inhibition of tumour growth in animal models. Further evaluation of its inhibitory activity at hybrid receptors (Hybrid-Rs) composed of the association between IGF-1R and insulin receptor (IR) was performed. Selective, potent and efficacious inhibition of [^{125}I]IGF-1 binding as well as IGF-1- and IGF-2-mediated receptor phosphorylation was demonstrated at both IGF-1R and Hybrid-Rs, without activity at IR. Ligand-independent down-regulation of both IGF-1R and Hybrid-Rs was obtained upon long-term association with h7C10. *In vivo* evaluation was performed in a MDA-MB-231 xenograft mouse model, showing a 14-fold higher level of Hybrid-Rs as compared to IGF-1R. A more potent anti-tumoural response was obtained for h7C10 as compared to Mabs targeting solely IGF-1R or Hybrid-Rs. The herewith described neutralising properties of h7C10 as potent inhibitor of both IGF-1R and Hybrid-Rs are likely to participate in its anti-tumoural activities and maybe of interest for therapeutic applications.

1. Introduction

The insulin-like growth factor-1 (IGF-1) receptor (IGF-1R) is a member of the receptor tyrosine kinase family. Numerous lines of evidence point to major and central implications of this receptor type in both tumour establishment and maintenance of a tumourigenic phenotype in animal models and human cancer (for recent reviews[1–3]). Therapeutic interventions, dealing with inhibition of IGF-1R signalling by various approaches such as antisense oligonucleotides,[4] tyrosine kinase inhibitors[5,6] and more recently monoclonal antibodies (Mabs,[7–14]), demonstrated efficient anti-tumoural activities both in *in vitro* experiments and in animal models. As a consequence of these studies, several of these compounds are actually under clinical investigations in humans.

The IGF-1R, as well as its close structural homologue, insulin receptor (IR), contains two α-and two β-chains, associated in a hetero-tetrameric functional structure *via* covalent disulphide bridges.[15] Human IR exists in two isoforms determined

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by alternative splicing of exon 11 located at the carboxy-terminus of the receptor α-subunit: isoform A (IR-A) lacks exon 11, whereas isoform B (IR-B) contains a 12 amino acid extension encoded by this small exon.[16] The relative expression of these two isoforms varies in a tissue-specific manner. Under conditions where IGF-1R is co-expressed with one of the IR isoforms, then hetero-tetrameric receptors can associate. Such hetero-tetramers formed by one IGF-1R α–β dimer and one α–β dimer from an IR are termed hybrid receptors (Hybrid-Rs,[17,18]). Hybrid receptors are formed particularly in cells and tissues expressing significantly different levels or overexpressing IGF-1R and IR, in which cases Hybrid-Rs can be the most represented subtype.[19,20] Similarly, Hybrid-Rs may also be overexpressed in a variety of tumour cells as a result of both IGF-1R and IR overexpression.[20,21] However, the biological role of these Hybrid-Rs remains largely unknown. When cancer cells express a high IR:IGF-IR ratio thereby yielding Hybrid-Rs, they also express a high Hybrid:IGF-IR ratio and predominantly contain IR hemidimers of the A isoform, referred as Hybrid-R-A.[20] Hybrid-R-A are receptors with a broad ligand specificity, being able to bind IGF-1 and IGF-2 with high affinity and also insulin albeit with a lower affinity.[18] Therefore, in malignancies with high Hybrid-R:IGF-1R ratio, IGF-1 and IGF-2 mostly signal through Hybrid-Rs rather than through homodimeric IGF-IR. Stimulation of IGF-1R and Hybrid-Rs by IGF-1 and IGF-2 results in the activation of various intracellular signalling cascades such as the IRS-1/PI3-Kinase/Akt pathway and the Shc/Ras/ERK1/2 pathway *via* protein phosphorylation, resulting in both proliferative and anti-apoptotic responses.

In the present study, we report functional aspects of Hybrid-Rs inhibition by a novel Mab (7C10) raised against IGF-1R and its humanised form (h7C10;[11]) and their possible relevance in anti-tumour activity. We previously reported that 7C10 and h7C10 act as potent and efficacious inhibitors of both IGF-1 and IGF-2-mediated tumour cell proliferation *in vitro* and are able to significantly reduce tumour size in xenografted mice.[11] We herein investigated in more details the functional properties of 7C10 and h7C10 with regard to Hybrid-Rs, as compared to a series of reference anti-IGF-1R antibodies [αIR-3,[22]; 1H7[7] and 47-9.[23]] We evaluated *in vitro* binding and functional receptor phosphorylation blockade and receptor down-regulation using various biochemical approaches such as competition radioligand binding, immunoprecipitation and Western blotting. Different cellular models, expressing both recombinant and native IGF-1R, IR and Hybrid-Rs were used. Functional relevance of the putative therapeutic potential of 7C10 and its humanised form was assessed in an *in vivo* xenograft model of MDA-MB-231 human breast cancer cells, showing a high Hybrid-Rs versus IGF-1R ratio. Strong Mab-mediated inhibition of ligand binding, ligand-mediated signaling and ligand-independent receptor down-regulation responses were observed for IGF-1R as well as for Hybrid-Rs. The efficient inhibitory activities of 7C10 and h7C10 at Hybrid-Rs in addition to IGF-1R, associated with their high degree of specificity and lack of agonist activity, are likely to be the key-properties involved in the potent *in vivo* anti-tumoural effect of these Mabs and underline their potential therapeutic interest.

2. Experimental procedures

2.1. Cells

R^- mouse fibroblasts were stably co-transfected to express either human IGF-1R or IR alone (referred to R^+ and R^-/IR cells, respectively) or both IGF-1R and IR of the A or B isoform, thereby expressing hybrid receptors A or B (Hybrid-R-A/B) as previously described.[18] A description of receptors and cells used in this study is presented in Table 1. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal bovine serum and containing 100 μg/ml hygromycin and 0.3 μg/ml puromycin.

2.2. Preparation of cell lysates

Cells grown until 80% confluence and serum starved for 24 h were washed three times with ice-cold PBS and lysed in cold binding buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.25% sodium deoxycholate, 10 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml pepstatin and 10 μg/ml leupeptin, pH 7.4. After the cells were scraped, samples were rotated for 15 min at 4 °C. Insoluble material was separated by centrifugation at 10,000*g* for 10 min at 4 °C. Protein concentration was determined by using a Bradford assay.

2.3. Ligand binding assay

Cell lysates were immunocaptured on Maxisorb plates precoated with 2 μg/ml of 17-69 anti-IGF-1R Mab for IGF-1R,[24] and 83-7 anti-IR Mab for IR and for Hybrid-Rs.[23] After washing, immunocaptured IGF-1R, IR or Hybrid-R were incubated with [^{125}I]IGF-1 (60 pM) or [^{125}I]insulin (60 pM) in 50 mM HEPES-buffered saline containing 0.05% Tween 20, 1% BSA, 2 mM sodium orthovanadate, 1 mg/ml bacitracin and 1 mM PMSF, pH 7.6, in the presence of increasing concentrations of unlabelled molecules. Non-specific binding was determined in the presence of 1 μM of unlabelled ligand. After 3 h incubation at room temperature, plates were washed and the radioactivity was counted in a gamma-counter.

2.4. Receptor immunoprecipitation

Cell lysates prepared as described above were used to immunoprecipitate IGF-1R, IR and Hybrid-R by incubation of the indicated Mab (4 μg) at 4 °C under constant rotation for 2 h and two additional hours with protein G-sepharose beads (Pharmacia, Uppsala, Sweden). Immunoprecipitates were eluted and subjected to SDS–PAGE (7.5% polyacrylamide) gel electrophoresis followed by immunoblotting. For cells containing only hIGF-1R, blots were revealed with an anti-IGF-1R β-subunit (Santa Cruz Biotechnology), all other cell types were revealed with an anti-IR β-subunit Mab (Santa Cruz Biotechnology).

2.5. Receptor phosphorylation

Cell lysates were prepared as described above and immunoprecipitated with 17-69 Mab. Following immunoprecipitation,

the pellet was resuspended in Laemmli sample buffer and subjected to SDS–PAGE (7.5% polyacrylamide) gel electrophoresis followed by immunoblotting with an anti-phospho-tyrosine Mab (R&D Systems, CA, US).

2.6. Receptor down-regulation

R^+/IR-A and R^+/IR-B cells were incubated with indicated Mabs in either the absence or presence of ligands for 24 h. Cells lysates were prepared as described above and subjected to immunoprecipitation and Western blot analysis with the indicated Mabs, as described above.

2.7. Assay of tumour growth in athymic nude mice

Non-ovariectomised female 6–8-week-old athymic Nude mice were housed in sterilised filter-topped cages, maintained in sterile conditions and manipulated according to French and European guidelines. MDA-MB-231, an oestrogen-independent breast cancer cell line, expressing low level of IGF-IR but a significant amount of hybrid receptors,[20] was selected for *in vivo* evaluations. Mice were injected sub-cutaneously (s.c.) with 10×10^6 MDA-MB-231 cells. Six days after cell implantation, tumours were measurable and animals were divided into groups of 12 mice with comparable tumour size. Mice were then treated intra-peritoneally (i.p.) with 0.5 mg/dose of either h7C10, αIR-3 or 47-9 antibodies twice a week. A control group injected with PBS was introduced as it has previously shown that no impact in tumour growth was observed when mice were injected with either a murine or a human IgG1 isotype control. The mice were followed for the observation of xenograft growth rate and body weight changes. Tumor volume was calculated by the formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$.

Table 1 – Description of receptor types and investigated cell lines

	Description
Receptors	
IGF-1R	Human insulin-like growth factor-1 receptor
IR-A	Human insulin receptor A isoform, lacking a 12 AA extension encoded by exon 11
IR-B	Human insulin receptor B isoform, containing a 12 AA extension encoded by exon 11
Hybrid-R-A	Hybrid receptor composed of one α–β portion of hIGF-1R and one α–β portion of IR-A
Hybrid-R-B	Hybrid receptor composed of one α–β portion of hIGF-1R and one α–β portion of IR-B
Cell lines	
R^-	3T3-like foetal fibroblasts derived from IGF-1R knock-out mice
R^+	R^- cells stably expressing human IGF-1R
R^-/IR-A	R^- cells stably expressing human IR-A
R^-/IR-B	R^- cells stably expressing human IR-B
R^+/IR-A	R^+ cells stably expressing IR-A, and containing Hybrid-R-A
R^+/IR-B	R^+ cells stably expressing IR-B, and containing Hybrid-R-B

2.8. Statistical method

Statistical significance of differences in tumour growth among the different treatment groups was determined by a Mann–Whitney *U* test using Statgraphics software.

2.9. Materials

The following materials were purchased from the indicated manufacturers: foetal calf serum, glutamine, LipofectAMINE and DNase I from Invitrogen (Paisley, UK); RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), minimum essential medium, Ham's nutrient mixture F-12, bovine serum albumin (BSA; radioimmunoassay grade), bacitracin, phenylmethylsulphonyl fluoride (PMSF), puromycin, bromodeoxyuridine (BrdUrd) and porcine insulin from Sigma; protein G-Sepharose from Amersham Biosciences (Uppsala, Sweden); and [^{125}I]IGF-1 and [^{125}I]insulin (specific activity of 11.1 MBq/μg) from PerkinElmer Life Sciences (Zaventem, Belgium). IGF-1 and IGF-2 were obtained from Calbiochem, and FuGENE 6 transfection reagent was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Athymic nude mice were from Harlan (Gannat, France).

The following anti-IR antibodies were employed: monoclonal antibodies MA-10 and MA-20 (Table 2 ; Dr. I.D. Goldfine, University of California at San Francisco, San Francisco, CA); monoclonal antibody 83-7 (Table 2; Dr. K. Siddle, University

Table 2 – List of investigated monoclonal antibodies and their specificities

Antibody designation	Reference/origin	Antigen	Antibody specificity
MA-10	[37]	Purified placental hIR	hIR
MA-20	[37]	Purified placental hIR	hIR
17-69	[17]	NIH-3T3/hIGF-1R	hIGF-1R = Hybrid-Rs
24-60	[17]	NIH-3T3/hIGF-1R	hIGF-1R = Hybrid-Rs
47-9	[23]	IM-9 lymphocytes	hIR = Hybrid-Rs
83-7	[23]	IM-9 lymphocytes	hIR = Hybrid-Rs
αIR3	[22]	Purified placental hIGF-1R	hIGF-1R ≫ Hybrid-Rs
1H7	[7]	Purified placental hIGF-1R	hIGF-1R
7C10	[11]	hIGF-1R ECD	hIGF-1R = Hybrid-Rs
h7C10	[11]	hIGF-1R ECD	hIGF-1R = Hybrid-Rs
IGF-1R-β-subunit	Upstate Biotechnology		β-Subunit-hIGF-1R = Hybrid-Rs
29B4	Santa Cruz Biotechnology		β-Subunit-hIR = Hybrid-Rs

of Cambridge, Cambridge, UK); and polyclonal antibody 29B4 (Table 2; Santa Cruz Biotechnology Inc., Santa Cruz, CA).

The following anti-IGF-IR antibodies were employed: monoclonal antibody αIR3 (Table 2; Oncogene Research, Cambridge, MA); monoclonal antibodies 17–69 and 24–60 (Table 2; Dr. K. Siddle) ; and a chicken polyclonal antibody that recognises the IGF-IR β-subunit (Upstate Biotechnology, Inc., Lake Placid, NY) ; anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology, Inc.

3. Results

3.1. *Competition radioligand binding with antibodies*

R^- cells, which do not express IGF-1R and a low amount of endogenous mouse IR, did not yield any detectable specific ligand binding, regardless of the investigated radioligand (not shown). On the other hand, experimental conditions based on receptor immunocapture from cell lysates were set up for detecting between 3000 and 13,000 cpm of specific binding using [^{125}I]IGF-1 and [^{125}I]insulin as radioligand.[18] IGF-1R-transfected R^+ fibroblasts were used for immunocapturing IGF-1R onto Maxisorb plates, as described in Section 2.9. Both natural ligands IGF-1 and IGF-2 potently displaced labelled IGF-1 from immunocaptured IGF-1R with mean IC_{50} values of, respectively, 2.1 ± 1.8 and 8.1 ± 4.5 nM (Fig. 1 and Table 3). Anti-IGF-1R Mabs 7C10 (mouse) and h7C10 (recombinant humanised) efficiently displaced [^{125}I]IGF-1 with similar affinities in the nanomolar range (Fig. 1 and Table 3). A widely studied reference anti-IGF-1R Mab, αIR3,[22] yielded a similar response with an IC_{50} value of 8.7 ± 4.6 nM. In contrast, 1H7, a mouse Mab directed against IGF-1R[7], was unable to displace [^{125}I]IGF-1 at concentrations up to 100 nM (Table 3), as it was the case for human IgG1 isotype control and for 9G4, a Mab directed against a protein unrelated to IGF-1R. Very similar results were obtained when competition studies of [^{125}I]IGF-1 binding were carried out on intact R^+ cells (data not shown).

Table 3 – IC_{50} values of a series of monoclonal antibodies (Mabs), polyclonal human IgG1 molecules and natural ligands at human IGF-1R immunopurified from stably transfected R^+ mouse fibroblasts

IGF-1R; IC_{50} values (nM)	
Ligand	[^{125}I]IGF-1
IGF-1	2.1 ± 1.8
IGF-2	8.1 ± 4.5
Insulin	> 100
7C10 Mab	6.9 ± 6.5
h7C10 Mab	6.9 ± 6.9
αIR3 Mab	8.7 ± 4.6
1H7 Mab	>100
9G4 Mab	>100
h IgG1 pool	>100

[^{125}I]IGF-1 (60 pM) binding experiments were performed on immunopurified hIGF-1R, extracted from membrane preparations of R^+ mouse fibroblasts as described in Section 2. Data were analysed graphically with inhibition curves and IC_{50} values (ligand concentration yielding 50% inhibition of specific [^{125}I]IGF-1) were derived.
Results are means ± SE mean of three to five independent experiments, each experimental data point was performed in duplicate.

In a next step, ligand binding properties were evaluated at hetero-tetrameric hybrid receptors. R^+ cells cotransfected with either IR-A or IR-B cDNAs (R^+/IR-A and R^+/IR-B cells) and expressing similar amounts of Hybrid-Rs[20] were used as a source of Hybrid-R-A and Hybrid-R-B, respectively. Both Hybrid-R subtypes bound equally IGF-1 and IGF-2 with high affinity (Fig. 2, Table 4). Mouse 7C10 and recombinant

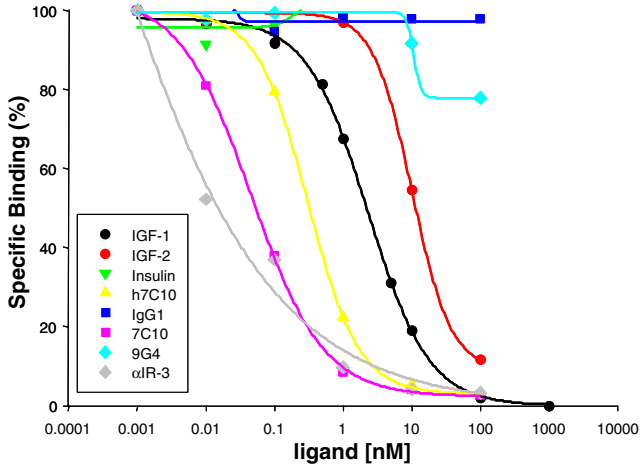

Fig. 1 – Inhibition curves of IGF-1R ligands, monoclonal antibodies and polyclonal human IgG1 pool to human IGF-1R. IGF-1R were immuno-captured with 17-69 Mab from R^+ mouse fibroblast membranes stably expressing IGF-1R as described in Section 2. Competition binding was performed using [^{125}I]IGF-1 (60 nM) as radioligand. This figure shows a representative experiment showing most potent inhibitory activities for h7C10 and 7C10 Mabs. Mean IC_{50} values are summarised in Table 3.

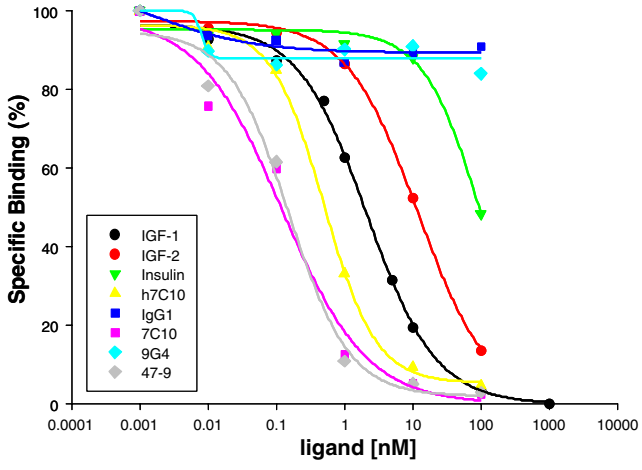

Fig. 2 – Inhibition curves of IGF-1R ligands, monoclonal antibodies and polyclonal human IgG1 pool to hybrid receptors Hybrid-R-A. Hybrid-R-A were immuno-captured with 83-7 Mab from R^+/IR-A mouse fibroblast membranes stably co-expressing IGF-1R and IR-A as described in Section 2. Competition binding was performed using [^{125}I]IGF-1 (60 nM) as a radioligand. This figure shows a representative experiment showing most potent inhibitory activities for h7C10 and 7C10 Mabs. Mean IC_{50} values are summarised in Table 4.

Table 4 – IC_{50} values of a series of monoclonal antibodies (Mabs), polyclonal human IgG1 molecules at hybrid receptors from A and B isoforms (Hybrid-R-A and Hybrid-R-B) immuno-purified from stably transfected R^+/IR-A and R^+/IR-B mouse fibroblasts

[^{125}I]IGF-1; IC_{50} values (nM)

Ligand	Hybrid-R-A	Hybrid-R-B
IGF-1	1.9 ± 1.1	1.7 ± 1.0
IGF-2	5.3 ± 4.0	3.7 ± 1.8
Insulin	35.7 ± 17.8	70.0
7C10 Mab	4.1 ± 3.9	3.0 ± 2.2
h7C10 Mab	6.5 ± 5.3	3.2 ± 1.3
47-9 Mab	3.0 ± 2.9	5.8 ± 4.3
1H7 Mab	>100	>100
9G4 Mab	>100	>100
h IgG1 pool	>100	>100

[^{125}I]IGF-1 (60 pM) binding experiments were performed on Hybrid-R-A and Hybrid-R-B extracted from membrane preparations of R^+/IR-A and R^+/IR-B fibroblasts and immunopurified with 83-7, as described in Section 2. Data were analysed graphically with inhibition curves and IC_{50} values (ligand concentration yielding 50% inhibition of specific radioligand binding) were derived.
Results are means ± SE mean of three to five independent experiments, each experimental data point was performed in duplicate.

humanised h7C10 Mabs efficiently displaced [^{125}I]IGF-1 from Hybrid-R-A (Fig. 2 and Table 4) and Hybrid-R-B (Table 4) with IC_{50} values in the low nanomolar range. Mab 47-9[23] corresponding to an anti-IR Mab recognising as well Hybrid-Rs[25] competed for [^{125}I]IGF-1 binding in a similar way (Table 4). The selective anti-IGF-1R Mab 1H7 as well as control Mabs 9G4 and human IgG1 isotype control were inactive at concentrations up to 100 nM towards both Hybrid-R-A and Hybrid-R-B (Table 4) subtypes.

Antibody specificity was assessed by studying competition with [^{125}I]insulin at R^- cells stably expressing IR (R^-/IR cells). None of the investigated anti-IGF-1R Mabs or reference Mabs were able to displace [^{125}I]insulin at its cognate receptors IR-A (Fig. 3) and IR-B (not shown) with exception of the control anti-IR Mab MA-10 (IC_{50}: 1.2 nM, Fig. 3).

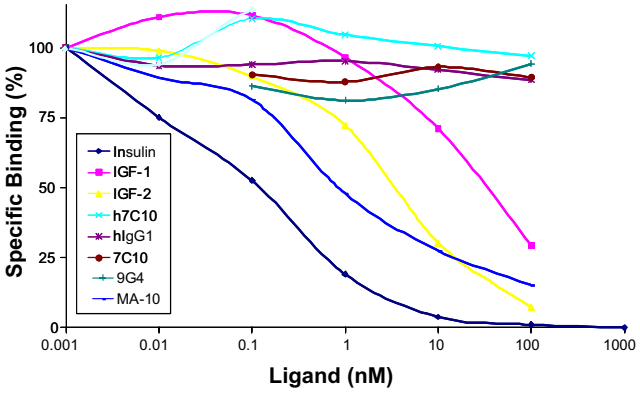

Fig. 3 – Inhibition curves of IR and IGF-1R ligands, monoclonal antibodies and polyclonal human IgG1 pool to insulin receptor A. IR-A were immuno-captured with 83-7 Mab from R^-/IR-A mouse fibroblast membranes stably expressing IR-A as described in Section 2. Competition binding was performed using [^{125}I]insulin (60 nM) as a radioligand. This figure shows one representative experiment out of three independent experiments.

3.2. *Inhibition of ligand-mediated receptor phosphorylation by antibodies*

In a first set of experiments, immunoprecipitation of either IGF-1R, Hybrid-Rs or IR was performed with a series of anti-IGF-1R and anti-IR Mabs. Both 7C10 and h7C10 recovered efficiently IGF-1R (Fig. 4, lanes 1 and 2) and Hybrid-Rs (Fig. 4, lanes 4, 5, 7, and 8), without yielding any IR precipitation (Fig. 4, lanes 10, 11, 13, and 14). In a next step, ligand-stimulated receptor phosphorylation and the inhibitory activity of Mabs were assessed. Both natural ligands IGF-1 and IGF-2 efficiently stimulated phosphorylation of the IGF-1R β-subunit (Fig. 5A, lanes 2 and 6) upon stimulation of cells for 15 min. The IGF-1- as well as IGF-2-mediated phosphorylation was efficiently inhibited by all three investigated Mabs (7C10, h7C10 and 1H7), although full inhibition was not observed (Fig. 5A, lanes 3–5 and 7–9). Inhibition of IGF-2-mediated IGF-1R phosphorylation was slightly less pronounced, although this difference was not reproducibly obtained. 1H7 Mab behaved as a partial agonist, generating by its own an IGF-1R phosphorylation signal (Fig. 5A, lane 10). Mabs 7C10 and h7C10 did not yield any receptor activation under these experimental conditions (Fig. 5 lanes 11 and 12).

In a similar way, both IGF-1 and IGF-2 efficiently stimulated Hybrid-R-A phosphorylation (Fig. 5B, lanes 2 and 6). The investigated Mabs (7C10, h7C10 and 1H7) inhibited both IGF-1 and IGF-2-mediated Hybrid-R-A phosphorylation (Fig. 5B, lanes 3–5 and 7–9). The efficiency of their inhibitory response seems comparable. Mabs 7C10 and h7C10 by their own were silent at Hybrid-R-A (Fig. 5B, lanes 11 and 12), whereas 1H7 Mab exhibited weak partial agonist activity

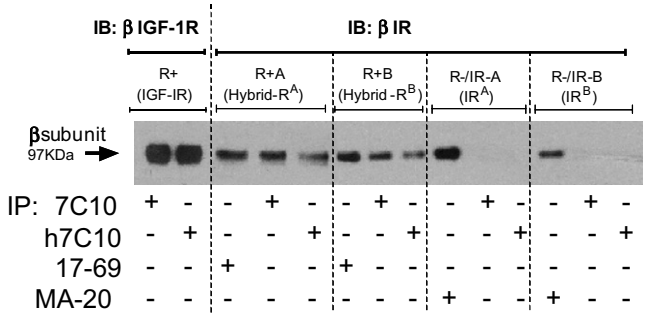

Fig. 4 – Immunoprecipitation of IGF-1R, Hybrid-Rs and IR by a series of anti-IGF-1R and anti-IR Mabs. IGF-1R, Hybrid-R-A, Hybrid-R-B, IR-A and IR-B were immunocaptured using the following transfected cells (see Table 1): R^+, R^+/IR-A, R^+/IR-B, R^-/IR-A and R^-/IR-B cells, respectively, using indicated Mabs. Cell lysates were prepared as described in Section 2. The pellet was then resuspended in Laemmli sample buffer and subjected to SDS–PAGE (7.5% polyacrylamide) gel electrophoresis followed by immunoblotting with anti-β-chain of IGF-1R (lanes 1 and 2) or anti-β-chain of IR (lanes 3–12) Mab. Arrow indicates position of the 97 kDa β-chain. This figure shows one representative

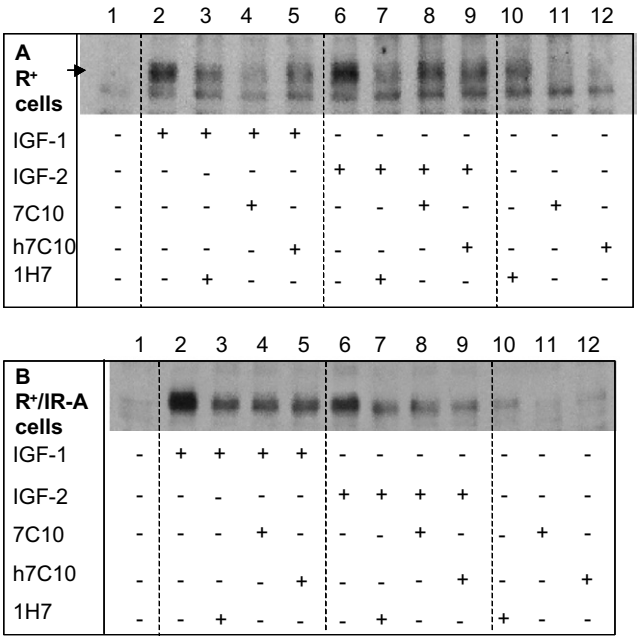

Fig. 5 – Inhibition of IGF-1 and IGF-2-mediated IGF-1R and Hybrid-R-A phosphorylation by a series of anti-IGF-1R Mabs. R^+ cells expressing IGF-1R (A) or R^+/IR-A cells containing Hybrid-R-A (B) were stimulated with IGF-1 (10 nM) or IGF-2 (10 nM) for a 15 min period in either the absence or presence of indicated Mab. Cell lysates were prepared as described in Section 2 and immunoprecipitated with 17-69 Mab. The pellet was then resuspended in Laemmli sample buffer and subjected to SDS–PAGE (7.5% polyacrylamide) gel electrophoresis followed by immunoblotting with an anti-phospho-tyrosine Mab. Arrow indicates position of the 97 kDa β-chain. This figure shows one representative experiment out of four independent experiments.

(Fig. 5B, lane 10). Similar results were obtained for Hybrid-R-B although a weaker phosphorylation signal by IGF-2 was observed, owing to its low binding affinity for Hybrid-R-B as compared to Hybrid-R-A (not shown).

3.3. *Antibody-mediated receptor down-regulation*

7C10 and h7C10 Mab-mediated IGF-1R down-regulation has already been demonstrated in MCF-7 cells, expressing moderate amounts of Hybrid-Rs.[11,20] We further evaluated Mab properties in a recombinant system showing elevated levels of Hybrid-Rs.[18] Neither IGF-1 nor IGF-2 affected the receptor expression level in R^+/IR-A and R^+/IR-B cells, independently of the investigated receptor type (Fig. 6A and B, lane 2; Fig. 6C and D, lanes 2 and 6) upon a 24 h stimulation. Both murine and humanised forms of 7C10 Mab reproducibly and fully down-regulated the level of IGF-1R both in the presence (Fig. 6A and B, lanes 3 and 4) and in the absence (Fig. 6A and B, lanes 7 and 8) of its cognate ligands IGF-1 or IGF-2. The reference antibody 24–60[17] (see Table 2) was as effective as h7C10 in down-regulating IGF-1R (Fig. 6A and B, lanes 5 and 9). Similar observations could be made for Hybrid-R-A (Fig. 6C) and Hybrid-R-B (Fig. 6D): their natural ligands [IGF-1, IGF-2 and insulin (not shown)] did not affect Hybrid-Rs expression after a 24-h stimulation. In contrast, Mabs 7C10 and h7C10 and the anti-Hybrid-R Mab 17–69[17](see Table 2) efficiently down-regulated Hybrid-R-A (Fig. 6C, lanes 3–5 and 7–9) and Hybrid-R-B (Fig. 6D, lanes 3–5 and 7–9), regardless of the presence or absence of ligands. Human IgG1 isotype control was without effect on receptor expression levels (Fig. 6A and B, lanes 6 and 10).

3.4. *Anti-tumour effects of h7C10, αIR3 and 47-9 antibodies on a MDA-MB-231 xenograft tumour model*

To determine the interest of targeting both IGF-1R and Hybrid-Rs *in vivo* in cells showing high Hybrid-Rs content, the h7C10 humanised antibody recognising both IGF-1R and Hybrid-Rs was compared to either the αIR3, a murine monoclonal antibody specific to the IGF-1R, or to the 47-9, a monoclonal antibody that binds to both IR and Hybrid-Rs but does not interact with IGF-1R. For that purpose, we set up a xenograft mouse model using oestrogen-independent MDA-MB-231 human breast cancer cells. This cell line was chosen because it is characterised by a high Hybrid-R content and a relatively lower IGF-1R content.[26] To ascertain the Hybrid-R:IGF-1R ratio, the content of these two receptor subtypes was measured by specific ELISAs:[20] Hybrid-Rs content was 69 ± 15 ng/mg protein while IGF-1R content was 5.0 ± 1.0 ng/mg protein, thus confirming a 14-fold Hybrid-R:IGF-1R ratio. Non-ovariectomised Swiss nude mice were injected s.c. with 10×10^6 MDA-MB-231 cells and randomised to twice a week i.p. treatment with 500 μg/injection of either h7C10, or αIR3 or 47-9 antibody. The antibody treatment was selected as an optimal dosing for both h7C10[11] and αIR3.[27] No *in vivo* data were available for 47-9 Mab. However, a schedule of 500 μg/injection twice a week can be considered at least as sub-optimal when compared to the dosing of various Mabs used *in vivo*. The control group was treated with PBS as we previously showed[11] that no difference in tumour growth was observed between mice receiving either PBS or non-related control antibodies. Twelve animals were included in each group. Treatment started 6 days after the injection of cells, when tumours were measurable. When mice were treated with the h7C10 Mab, a significant anti-tumour activity ($p \leqslant 0.01$ from day 12 to day 37) was observed in the MDA-MB-231-grafted mice (Fig. 7). Average tumour volume at 6 weeks post-cell injection was reduced by 64%. In contrast to that observed with h7C10, treatment with αIR3 slightly inhibited MDA-MB-231 *in vivo* growth with a significant tumour volume inhibition observed only between day 12 and day 14 ($p \leqslant 0.03$, Fig. 7). For 47-9 Mab, an intermediate significant inhibitory effect was observed between day 12 and day 26 ($p \leqslant 0.04$, Fig. 7).

4. Discussion

In the present report, we evaluated the ability of a novel anti-IGF-1R monoclonal antibody, 7C10 and its recombinant humanised, CDR-grafted form, h7C10, to functionally interfere with the signalling of both IGF-1R and Hybrid-Rs composed of IGF-1R and IR hetero-dimers. Both murine and humanised forms have been shown to have potent and

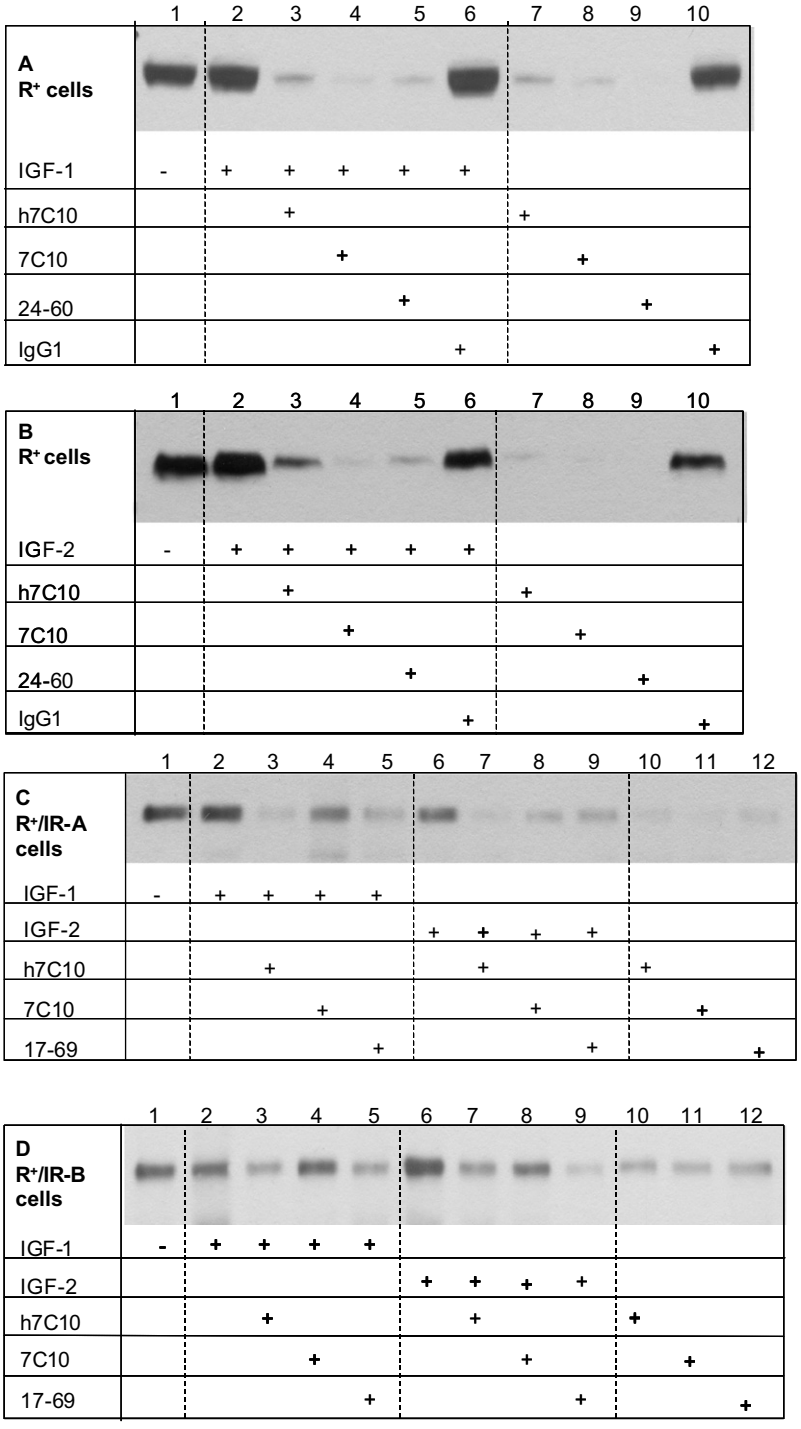

Fig. 6 – Antibody-mediated IGF-1R and Hybrid-R-A down-regulation by a series of anti-IGF-1R Mabs. R^+ cells expressing IGF-1R (A,B), R^+/IR-A cells containing Hybrid-R-A (C) or R^+/IR-B cells containing Hybrid-R-B (D) were stimulated with IGF-1 (10 nM, A,C,D) or IGF-2 (10 nM, B,C,D) for a 24 h period in either the absence or presence of indicated Mab. Cell lysates were prepared as described in Section 2 and immunoprecipitated with 17-69 Mab for IGF-IR or 83-7 for Hybrid-Rs. The pellet was then resuspended in Laemmli sample buffer and subjected to SDS–PAGE (7.5% polyacrylamide) gel electrophoresis followed by immunoblotting with an anti-IGF-IR Mab. This figure shows representative experiments for each receptor type out of three to four independent experiments.

efficacious inhibitory activities in various *in vitro* and *in vivo* tumour models.[11] Salient results obtained with these antibodies include the following: (i) h7C10 inhibited in the nanomolar range both IGF-1 and IGF-2-induced proliferation of the oestrogen-dependent MCF-7 human breast cancer cell line by blocking cell cycle progression, (ii) h7C10 inhibited IGF-1-induced signalling with decreased IGF-1R β-chain and IRS-1 phosphorylation, (iii) treatment of nude mice bearing either

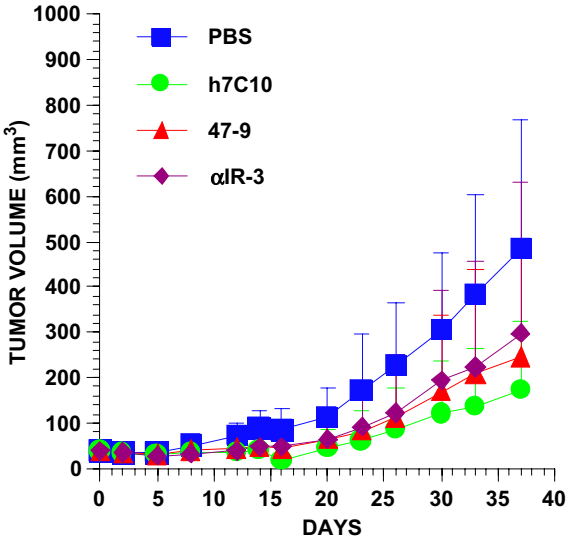

Fig. 7 – Anti-tumour activity of h7C10, αIR3 and 47-9 antibodies upon an established xenograft tumour model. Athymic nude mice (n = 12) were engrafted s.c. with 10×10^6 MDA-MB-231 cells. Six days after engraftment, mice were treated i.p. with a loading dose of 1 mg of antibody/mouse and then twice weekly with 500 μg/mouse. Control group received PBS. h7C10 significantly inhibited tumour growth until day 12 ($p \leqslant 0.01$), whereas αIR3 showed a significant activity only on days 12 and 14. The 47-9 Mab had an intermediate activity with a significant inhibition of tumour growth between day 12 and day 26 ($p \leqslant 0.04$).

human breast cancer (MCF-7) or lung cancer (A549) cells with h7C10 significantly inhibited tumour growth, (iii) combined therapy with h7C10 and either the chemotherapeutic agent Vinorelbine or an anti-EGFR Mab (225) significantly prolonged life span of mice in an orthotopic grafting model of A549 cells.[11]

The possible relevance of Hybrid-Rs in cancer biology has been highlighted by recent findings that the IR and especially its short isoform IR-A is often overexpressed in human malignancies.[28,29] By using specific ELISAs, we were able to measure Hybrid-R content in human breast cancer cell lines as well as breast tissue samples and showed that it exceeded the IGF-1R content in more than 75% of breast cancer samples[20]. This high content of Hybrid-Rs in cancer results by the frequent co-expression of high levels of both IR and IGF-1R.[20] In fact, Hybrid-R formation occurs by random assembly of receptor hemidimers,[30] although preferential assembly of Hybrid-Rs seems also to occur in certain cancers.[31] A high Hybrid-R:IGF-1R ratio is often observed in poorly differentiated carcinomas.[32] Hybrid-Rs behave as functional IGF-1Rs,[24] therefore, Hybrid-R overexpression produces additional functional receptors that respond to IGF-1. This is particularly true for Hybrid-R incorporating IR-A moieties (Hybrid-R-A), which show a higher binding affinity than Hybrid-R-B with regard to IGF-1, IGF-2 and insulin.[18] Besides cancer, a functional role of Hybrid-Rs has also been reported in diseases of the central nervous system, such as scrapie infection.[33] Taken together, these observations clearly indicate that potential therapies designed to block the functional consequences of IGF-1R ligands must take into account the role of Hybrid-Rs, and that powerful therapies must block or interfere not only with IGF-1R but also with Hybrid-Rs thereof.

Accordingly, we have previously shown *in vitro* evidence that a considerable proportion of the mitogenic effects of IGF-I can be inhibited by blocking the binding of IGF-I to Hybrid-Rs in cancer cells with a high Hybrid-R:IGF-IR ratio, as the IGF-I mitogenic effect occurs predominantly *via* the receptor type that is the most abundant.[20] Herein, we show by competition binding studies that both 7C10 and h7C10 directly compete for [^{125}I]IGF-1 binding at both IGF-1R and Hybrid-R without recognition of IR and interference with insulin binding. This binding feature is likely to prevent downstream IGF-1R and Hybrid-Rs phosphorylation, either directly or by inducing a conformation shift and thereby constraining the receptor in an inactive conformation, unable to activate the cytoplasmic tyrosine kinase domain and inhibiting subsequent phosphorylation events.

Receptor down-regulation and the putative receptor degradation that may follow are important properties to consider for an antagonist antibody. Indeed, continuous exposure of an antibody that only displaces the ligand would be required for efficient anti-tumoural activity, while this continuous exposure is not needed if the antagonist antibody causes degradation and depletion of target receptors from the cell surface. It can be anticipated that IGF-1R and Hybrid-Rs down-regulation by specific antibodies will blunt IGF-1 and IGF-2-mediated downstream receptor signalling cascades and proliferation of the targeted cell types. As expected, anti-proliferative and/or anti-tumoural responses have been obtained with anti-IGF-1R Mab causing IGF-1R degradation in both *in vitro* and *in vivo* cancer models: this was the case of the 4G11 Mab[12] and the MAB391 Mab.[34] Down-regulation mediated by 7C10 and h7C10 was selective for IGF-1R and Hybrid-Rs. This is in contrast with the heterologous down-regulation mediated by EM164 and its derived scFv fragment: these molecules, although not reacting against IR, were able to down-regulate IR levels in MCF-7.[35] This non-specific down-regulation was speculated to involve Hybrid-Rs. This discrepancy may be related to the mechanism of receptor down-regulation and degradation induced by these Mabs: lipid rafts-directed IGF-1R down-regulation was demonstrated for EM164 and its scFv,[35] the mechanism of receptor down-regulation mediated by 7C10 and h7C10 is still unknown but can be different, involving for example the ubiquitin–proteasome degradation pathway.

To evaluate the potential benefit of targeting both IGF-1R and Hybrid-Rs by 7C10, we set up an *in vivo* experiment using antibodies displaying different patterns of recognition of these receptors such as αIR3, described as specific for IGF-1R,[22] and 47-9, an anti-IR Mab that also recognises Hybrid-Rs and strongly inhibits the IGF-1-induced stimulation of cells in an *in vitro* proliferation assay.[20] Many breast cancer cell lines have been described to express a high Hybrid-R:IGF-IR ratio. Among them we chose oestrogen-independent MDA-MB-231 cells, which confirmed to have a high Hybrid-R content, approximately 14-fold higher than IGF-1R content, as determined by specific ELISAs. MDA-MB-231 cells were xenografted in nude mice and used to compare the relative

efficiency of h7C10, αIR3 and 47-9 antibodies in inhibiting *in vivo* tumour growth. We found that h7C10 was able to significantly inhibit MDA-MB-231 *in vivo* growth as already observed for an oestrogen-dependent breast cancer model (MCF-7;[11]). Interestingly, αIR3, which is unable to recognise Hybrid-Rs, failed to significantly impair tumour progression suggesting that the neutralization of Hybrid-Rs in addition to IGF-1R could be an advantage in such a model. The statistically significant albeit lower activity observed with the 47-9 antibody seems to be in agreement with this hypothesis. To our knowledge, this is the first *in vivo* evidence that a Mab that blocks both IGF-IR and Hybrid-Rs can be more efficacious than the one blocking only IGF-IR, at least as far as cancer cells with high Hybrid-R:IGF-IR ratio are concerned. We cannot exclude, however, that other beneficial features of h7C10, such as its lack of agonistic activity as compared to αIR-3, may have contributed to this result.

In conclusion, important properties have been demonstrated for h7C10. Firstly, this Mab is highly specific for binding to IGF-1R and Hybrid-Rs, which are often the most represented receptor subtypes of the IGF system in human malignancies, without affecting IR. Secondly, it displays a potent and efficacious capacity to functionally inhibit both IGF-1R and Hybrid-Rs, and to induce their down-regulation and degradation. Thirdly, it does not exhibit any agonistic activity, unlike other similar antibodies. Finally, the demonstration of potent anti-tumoural activity in a mouse model further supports the benefit of targeting Hybrid-Rs, in addition to IGF-1R, in cancer cells with a high Hybrid:IGF-IR ratio. The evaluation of the Hybrid-R:IGF-IR ratio may prove to be a useful predictor of the clinical response of anti-IGF-1R therapeutic antibodies with different affinity to the various receptor subtypes. Interestingly, these inhibitory properties of h7C10 on Hybrid-Rs may possibly extend to other heterologous associations of receptors involving IGF-1R, such as the recently described IGF-1R/HER-2 hetero-dimers in herceptin-resistant tumour cells.[36]

Conflict of interest statement

Disclosed conflicts of interest include employment (T.W., B.A., N.C., L.G.) and, Contract Research Grant from Institut de Recherche Pierre FABRE (G.P., A.B.).

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