EUROPEAN JOURNAL OF CANCER 46 (2010) 3251–3262

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Anti-insulin-like growth factor 1 receptor antibody EM164 (murine AVE1642) exhibits anti-tumour activity alone and in combination with temozolomide against neuroblastoma ☆

Birgit Geoerger [a,*], *Jean-François Brasme* [a], *Estelle Daudigeos-Dubus* [a], *Paule Opolon* [b], *Corinne Venot* [c], *Laurent Debussche* [c], *Patricia Vrignaud* [c], *Gilles Vassal* [a]

[a] *UPRES EA 3535, Pharmacology and New Treatments of Cancer, Université Paris-Sud XI, Institut Gustave Roussy, 94805 Villejuif, France*
[b] *UMR 8121 Vectorology and Gene Transfer, Institut Gustave Roussy, 94805 Villejuif, France*
[c] *sanofi-aventis, Oncology Therapeutic Department, Centre de recherche de Vitry-Alfortville, 94493 Vitry/Seine CEDEX, France*

ARTICLE INFO

Article history:
Received 7 May 2010
Received in revised form 28 May 2010
Accepted 2 June 2010
Available online 28 June 2010

Keywords:
Insulin-like growth factor pathway
Monoclonal antibodies/therapeutic use
Pediatric cancer
Sympathic nervous system tumours
Alkylating agents
Retinoid acid

ABSTRACT

Insulin-like growth factor 1 receptor (IGF-1R) is overexpressed in many tumours and contributes to tumourigenicity, cell proliferation, metastasis and resistance, thus representing a promising therapeutic target. The human IGF-1R antagonistic monoclonal antibody EM164 (murine AVE1642) has shown activity in adult cancers and is being evaluated in patients with advanced malignancies. We investigated the EM164 for its therapeutic potential against childhood neuroblastoma.

EM164 at 0.07, 0.7 and 7 μg/mL exhibited anti-proliferative activity against all nine cell lines tested in ^{3}H-thymidine incorporation assay *in vitro*. Cell proliferation after EM164 exposure ranged between 24% and 80% compared to controls. Sensitivity was independent from culture serum conditions, intensity of IGF-1R expression and IGF-II secretion, although associated with inhibition of AKT activation. *In vivo*, EM164 administered intravenously at 40 mg/kg twice weekly for 4 weeks yielded significant tumour growth delays (TGD) of 13.4 d in advanced stage IGR-N91 and 12.9 d in SK-N-AS tumours compared to controls ($p = 0.02$ and $p = 0.0059$, respectively). Simultaneous treatment of EM164 0.7 μg/mL and temozolomide resulted in enhanced activity *in vitro*. *In vivo*, treatment with temozolomide at the maximum tolerated dose (100 mg/kg/d for 5 consecutive days) and EM164 yielded a significantly greater TGD of 29.1 d ($p < 0.01$) and two complete tumour regressions (CR) compared to 18.1 d (p = ns) and one CR for EM164 alone and 16.1 d (p = ns) for temozolomide alone.

Our results demonstrate the potential of the anti-IGF-1R antibody alone and in combination with alkylating agents and support the therapeutic development of the AVE1642 for aggressive neuroblastoma.

☆ The work was presented in part at the 97th Annual Meeting of the American Society of Cancer Research in Washington, DC, USA, in 2006 (Proceedings of the AACR 2006;1222) and at the 20th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics in Geneva, Switzerland, in 2008 (Eur J Cancer 2008;79(Supplements):562) and supported by Sanofi-Aventis, Vitry/Seine, France. J.F.B. was supported by a grant of the Association pour la Recherche sur le Cancer (ARC).

* *Corresponding author:* Address: Institut Gustave Roussy, UPRES EA 3535, Pharmacology and New Treatment of Cancer, 39 Rue Camille Desmoulins, 94805 Villejuif, France. Tel.: +33 1 42 11 46 61; fax: +33 1 42 11 52 75.
E-mail addresses: geoerger@igr.fr, sara.calmanti@igr.fr (B. Geoerger).

0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.06.005

1. Introduction

Dysregulation of type 1 insulin-like growth factor receptor (IGF-1R) pathway has been involved in promoting oncogenic transformation, cell proliferation, metastasis, angiogenesis and resistance in numerous cancers, such as multiple myeloma, breast, prostate, colon, ovarian, lung carcinomas[1,2] and neuroblastoma.[3] The receptor mediates proliferation when activated by the stimulatory ligands IGF-I and IGF-II. In normal cells, activation of IGF-1R, as well as the insulin receptor (IR), is tightly regulated by the action of IGF-binding proteins (IGFBP) and the non-stimulatory receptor IGF-IIR. IGF-1R activation results in proliferation through activation of the major adaptor protein insulin receptor substrate 1 (IRS-1), extracellular signal-related kinase (ERK), nuclear accumulation of cyclin D1 at the G1-S transition, and antagonises apoptotic cell death via the mitochondrial pathway by activating the serine/threonine kinase AKT.[4]

IGF-1R has been a major focus for the development of various novel anticancer therapies.[5] Most strategies target IGF-1R and not IR, due to potential metabolic consequences of inhibiting the latter. The antagonistic monoclonal antibody EM164 (murine version of the humanised AVE1642) binds specifically to the human IGF-1R with a Kd of 0.1 nM which is very close to the receptor Kd of 0.16 nM for IGF-I,[6] without binding to the IR. EM164 inhibits receptor activation, IGF-1R-mediated cell growth and survival.[7] *In vivo* efficacy of EM164 was shown in an IGF-1R-dependent murine embryo fibroblast model expressing the human *IGF1R* gene, in human BxPC-3 pancreatic, and HT29 colon cancer models (Bladt et al., unpublished data).

Evidence indicates that IGF-1R function is important in the pathogenesis of embryonal cancers, such as neuroblastoma.[8] Growth factors are essential during embryonal development and are usually down-regulated after birth. Most embryonal tumours highly express the receptor as well as its ligands. Neuroblastoma cell lines secrete and respond to IGF-II[9] and primary neuroblastoma specimens express IGF-II RNA and IGF-1R.[10] IGF-I stimulates chemotaxis and migration of neuroblasts through IGF-1R activation and PI3K signalling.[11] IGF-1R expression regulates neuroblastoma metastasis to bone.[12] IR-IGF-1R coexpression correlated with enhanced apoptosis and dedifferentiation.[13] IGF-1R overexpression promotes neuroblastoma cell survival by preventing mitochondrial membrane depolarisation and caspase-3 activation.[14] Furthermore, a transcriptional regulation of IGF-1R through *MYCN* gene expression has been suggested,[15] and *MYCN* expression could be disrupted by IGF-1R blockade with the antibody alphaIR3.[16] Different therapeutic approaches that inhibit IGF-1R signalling have been reported to reduce neuroblastoma cell growth: antisense to the IGF-1R,[17] nordihydroguaiaretic acid (NDGA),[18] the monoclonal antibody alphaIR3,[16,19] tyrosine kinase inhibitors NVP-AEW541[20] and BMS-554417.[21]

This study evaluated the murine antibody of AVE1642, EM164, in childhood neuroblastoma cell lines and xenografts alone and in combination with the alkylating agent temozolomide and the differentiating agent cis-retinoic acid.

2. Material and methods

2.1. Drugs

EM164 (murine AVE1642; produced by Immunogen and provided by sanofi-aventis) was stored diluted in serum-free medium for use *in vitro* or phosphate-buffered solution (PBS) *in vivo*. Temozolomide pure powder (Sigma–Aldrich Chimie SARL, Lyon, France) was diluted in dimethylsulphoxide (DMSO) or resuspended in hydroxypropyl cellulose (Klucel, Hercules-Aqualon, Le Pecq, France), respectively. 13-Cis retinoic acid (RA; Sigma–Aldrich Chimie) was dissolved in DMSO.

2.2. Cell lines and xenografts

SH-SY5Y, SK-N-AS, SK-N-SH, SK-N-BE, LAN-1, LAN-5 and IMR32 neuroblastoma cell lines were provided by Dr. Valent (Institut Gustave Roussy). IGR-NB8 was established from a primary stage 3 abdominal neuroblastoma,[22] IGR-N91 from a primary bone marrow metastasis.[23] LAN-1, LAN-5 and IMR32 cells were maintained in Roswell Park Memorial Institute (RPMI) medium, all others in Dulbecco's Modified Eagle's Medium (DMEM), containing 10% foetal calf serum (FCS; all Invitrogen SARL, Cergy Pontoise, France) at 37 °C and 5% CO_2. All cell lines were free of mycoplasma.

2.3. Cell proliferation ^{3}H-thymidine incorporation assay

Cells were plated in 96-well plates at 1.5×10^4/well in 10% FCS-conditioned medium and allowed to adhere overnight. After 7 h culture in 1% or 10% FCS medium, cells were exposed to EM164 at 7, 0.7 and 0.07 μg/mL in triplicates during 48 h. One mCi ^{3}H-thymidine (Amersham Bioscience Europe, Orsay, France) was added; plates were frozen at −20 °C. Cells were harvested onto GF/C filter (UniFilter®, Perkin–Elmer) using a Packard FilterMate™ Harvester and the incorporated radioactivity was determined using the TopCount® Microplate Scintillation Counter (Packard BioScience Company). Percentage of proliferation rate between treated and untreated wells expresses the means of 3–4 independent experiments.

2.4. MTT cell proliferation assay

Cells seeded in 96-well plates at $0.75–2.0 \times 10^4$/well in 10% FCS medium were treated with EM164 at 0.7 μg/mL, temozolomide at 200, 400 or 800 μM and 13 cis-retinoic acid at 10 μM. Cell viability was determined after 48 h exposure using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and lysis solution (20% sodium dodecyl sulphate–dimethylformamide) by colorimetric measurement at 620 nm in microplate reader (Multiskan, Thermo Electron Corporation, Courtaboeuf, France). Percentage of viability rate between treated and untreated wells expresses the means of at least three independent experiments.

2.5. IGF-1 receptor quantification by flow cytometry

Cells were cultured in 10% FCS medium, harvested using cell dissociation solution (Sigma–Aldrich Chimie), incubated with

or without 50 μg/mL EM164 per 0.5×10^6 cells during 30 min at 4 °C, followed by 20 μg/mL GAM-FITC for 30 min and resuspended in PBS containing 10 μg/mL propidium iodide to exclude dead cells. QIFIKIT (DAKO SAS, Trappes, France) beads with known quantities of mouse IgG were used to establish a calibration curve and to determine the mean molecules of equivalent fluorochrome (MEF) for the cells according to the manufacturer's instructions.

2.6. Cell cycle analysis by flow cytometry

Cells were treated in exponential growth with EM164 0.7 μg/mL, harvested at 24, 48 and 72 h and stained with propidium iodide buffer (0.002% propidium iodide, 20 mM ethylenediaminotetraacetic acid (EDTA) and 0.01% RNase). DNA content of individual cells was measured using a fluorescence-activated cell sorter (FACS)calibur and analysed with the Cell Quest program (BD Biosciences, Erembodegem, Belgium).

2.7. Western blot analysis

Cells were cultured in 10% FCS conditions, treated with EM164 0.7 μg/mL and harvested at 2, 6, 24 and 48 h. Cell lysates were isolated with lysis buffer TNEN (Tris NaCl EDTA NP40) containing 250 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA (pH 8.0), 0.1% Nonidet-P-40, anti-protease (Complete mini, Roche Diagnostics, Meylan, France) and anti-phosphatases (Na_3VO_4 1 mM, NaF 50 mM; Sigma–Aldrich Chimie) and homogenised with a potter Teflon-glass homogeniser. Protein amounts were quantified using the Micro BCA protein assay kit (Pierce, Perbio, Brebieres, France). Equal amounts of protein were separated electrophoretically in 7% sodium dodecyl sulphate (SDS)–PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond P; Amersham Bioscience Europe). Immunodetection was performed using the monoclonal mouse antibodies anti-β-actin (1:5000; AC-15; Sigma–Aldrich, St Quentin-Fallavier, France), anti-poly(ADP-ribose) polymerase 1 (1:200; PARP; C-2-10; Calbiochem, USA), rabbit polyclonal anti-human IGF-1Rβ (1:200; sc-713; Santa Cruz Biotechnology, Tebu-Bio SA, Le Perray en Yvelines, France), anti phospho-IGF-1Rβ (Tyr1131)/insulin receptor β (Tyr1146), anti-human AKT, phospho-AKT (Ser473), p44/42 MAPK (Erk1/Erk2), phospho-p44/42-MAPK (Thr202/Tyr204) and caspase-3 (clone #9662), all diluted 1:1000 (Cell Signaling Technology®, Ozyme, St Quentin-en-Yvelines, France). Blots were revealed with peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody, respectively, followed by ECL Plus chemiluminescence solution (Amersham Bioscience).

2.8. Animals

Female Swiss athymic mice (SPF – Specific Pathogen Free) were bred in the Animal Experimentation Unit at Institut Gustave Roussy. Experiments were carried out under the conditions established by the European Union (Directive 86/609/CCE).

2.9. Experimental in vivo design

Anti-tumour activity in advanced stage tumours was evaluated as previously described.[22] For IGR-N91 xenografts, tumour fragments (30 mm^3) were xenotransplanted subcutaneously in athymic mice 6–8 weeks of age. SK-N-AS xenografts were established through subcutaneous injection of 5×10^6 SK-N-AS cells. On day 0 of treatment, animals bearing a total tumour burden of 100–300 mm^3 were randomly assigned to treatment groups. EM164 was administered intravenously at 40 mg/kg twice weekly and temozolomide orally at 100 mg/kg in 0.2 mL Klucel solution for 5 d. Body weight and two tumour perpendicular diameters were measured two to three times weekly with a caliper. Each tumour volume was calculated according to the equation V (mm^3) = width^2 (mm^2) × length (mm)/2. The experiments lasted until tumour volumes reached 1500–2000 mm^3. Tumour doubling time (Td) was determined in an exponential growth phase between 200 and 400 mm^3. Tumour growth delays (TGD) were defined as T–C: where T and C were the median times (in days) required for the treatment and the control group tumours, respectively, to reach a predetermined size (750 mm^3). This allowed the quantitation of the tumour cell kill. The total log cell kill was calculated according to the following formula: total log CK = (T–C in days)/(3.32 × Td) and converted to an arbitrary activity rating according to the Southern Research Institute (SRI) criteria: highly active ++++ = total log cell kill (treatment duration of 5–20 d) > 2.8, +++ = 2.0–2.8, ++ = 1.3–1.9, + = 0.7–1.2, inactive − ⩽ 0.7. Complete tumour regression was defined as total regression or tumour volume < 63 mm^3 in two consecutive measurements. Statistical significance was determined using the two-tailed non-parametric Mann–Whitney or Kruskal–Wallis test and Prism® software version 3.00.

2.10. Histology and immunohistochemistry (IHC)

Animals bearing SK-N-AS xenografts were treated with EM164 40 mg/kg on days 0 and 3 and sacrificed 24 h after the second EM164 administration. Tumours were fixed in FineFix® (Milestone, Bergamo, Italy), paraffin embedded and cut into microsections. Sections were stained with haematoxylin–eosin–safranin (HES) for morphology. Immunodetection was performed after microwave pre-treatment in citrate buffer (pH 6.0) using the rabbit antibodies anti-human p-AKT (Ser473), diluted 1:50, and anti-human caspase-3, diluted 1:100 (both Cell Signaling), revealed by the rabbit PowerVision kit (ImmunoVision Technologies, CA) and diaminobenzidine.

3. Results

3.1. EM164 exhibits anti-proliferative activity in neuroblastoma cells

EM164 treatment at 0.07, 0.7 and 7 μg/mL resulted in reduced cell proliferation of all nine neuroblastoma cell lines as measured by ^{3}H-thymidine incorporation (Fig. 1A; Supplementary Table). IGR-NB8 and SK-N-BE were less sensitive with proliferation rates of 50% and 54%, respectively, at 7 μg/mL. The other cell lines exhibited proliferation rates between 24% and 39% at 7 μg/mL. LAN-5, IMR32, IGR-NB8 and SH-SY5Y were equally sensitive in serum and serum-deprived conditions. LAN-1, SK-N-AS and SK-N-SH showed a 15–20% reduced, SK-N-BE and IGR-N91 an increased proliferation rate under culture in serum-reduced conditions. No dose-dependent effect was ob-

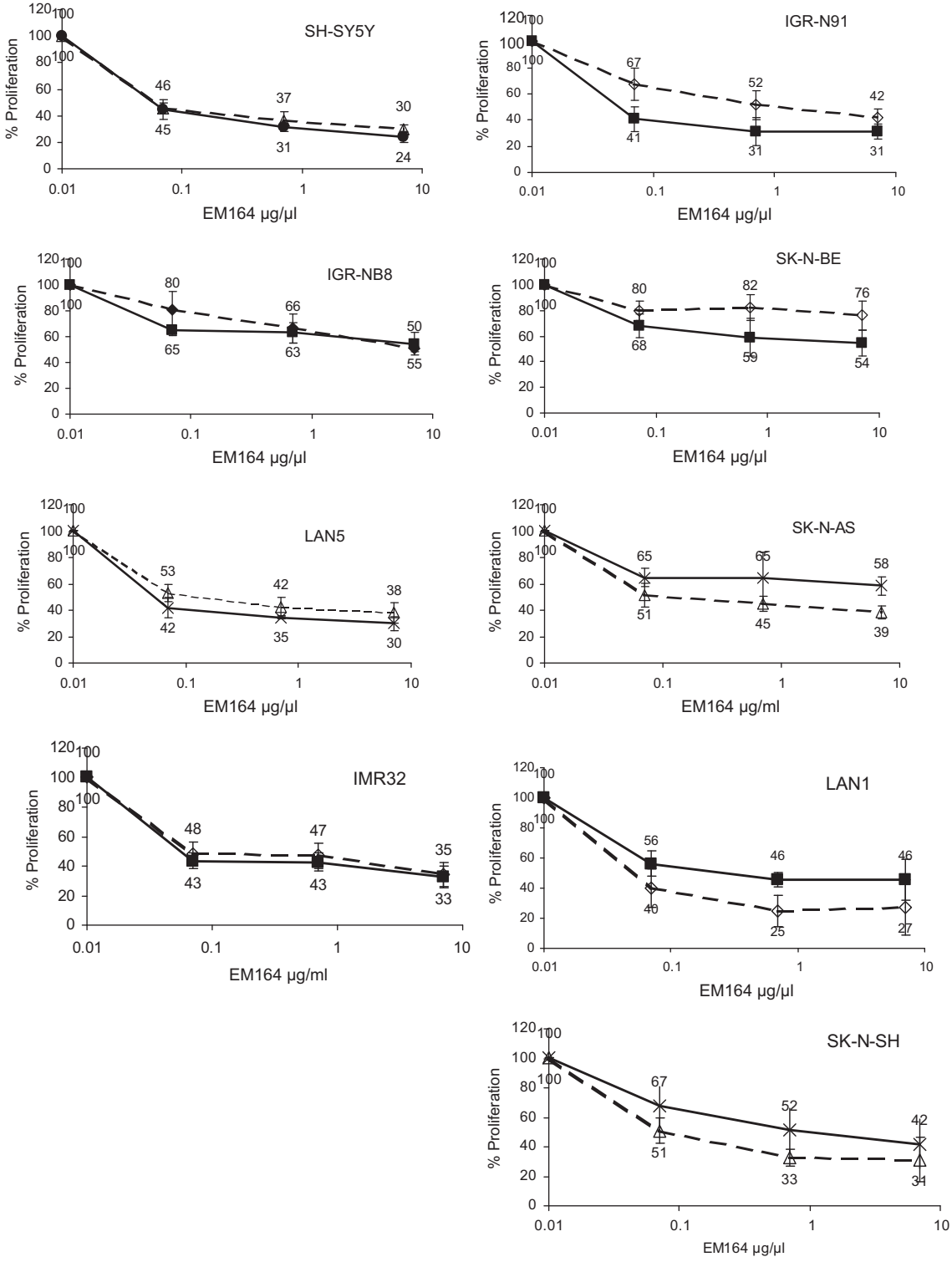

Fig. 1A – Anti-proliferative activity of EM164 in neuroblastoma cell lines. Cell proliferation of neuroblastoma cell lines cultured in 10% (continuous line) and 1% (intermittent line) FCS-conditioned medium after 48 h exposure to EM164 treatment at 0.07, 0.7 and 7 µg/mL as measured by ^{3}H-tymidine incorporation. Data represent means out of 4–5 independent experiments each performed in triplicates. Sensitivity was not systematically dependent on serum culture conditions.

served since all the three antibody concentrations chosen were saturated.[24] EM164 sensitivity was independent from the *MYCN* gene status with SK-N-AS, SK-N-SH and SH-SY5Y being non-amplified and all others exhibiting an *MYCN* gene amplification. Thus, neuroblastoma cells are highly sensitive to EM164 *in vitro*.

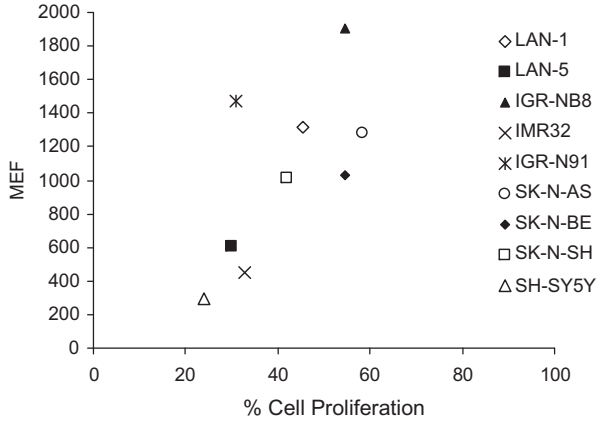

Fig. 1B – EM164 sensitivity *in vitro* is independent from levels of IGF-1R expression. Sensitivity of neuroblastoma cells to EM164 is expressed as percentage of cell proliferation following EM164 0.7 mg/mL treatment and cultured in 10% serum conditions as measured by ^{3}H-thymidine incorporation. For quantitation of IGF-1R, neuroblastoma cells were stained with EM164 and receptor expression level was quantified with the help of the QIFIKIT standards and flow cytometry and expressed in mean molecules of equivalent fluorescence (MEF).

3.2. *EM164 sensitivity in neuroblastoma cells is independent of IGF-1R expression*

We intended to explore if *in vitro* sensitivity is correlated with IGF-1R expression. IGF-1R was found expressed in all 9 neuroblastoma cells (IGR-NB8: 1904 median equivalent of fluorescence (MEF), IGR-N91: 1469, LAN-1: 1317, SK-N-AS: 1280, SK-N-BE: 1028, SK-N-SH: 1015, LAN-5: 602, IMR32: 451, SH-SY5Y: 294). IGF-1R levels were not correlated with *in vitro* sensitivity to EM164 as determined by percentage cell proliferation and shown for 7 μg/mL of EM164 and 10% FCS condition

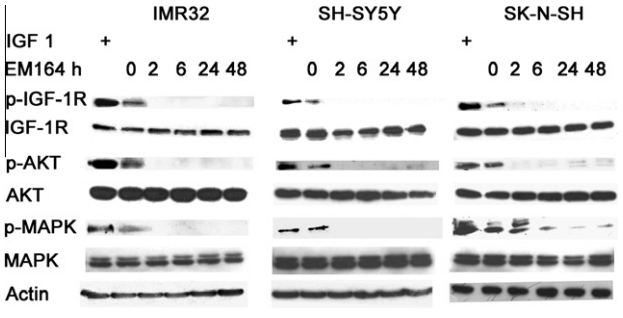

Fig. 2 – EM164 inhibits IGF-1R, AKT and MAPK activation in neuroblastoma cells. Neuroblastoma cells were cultured in 10% FBS-conditioned medium and harvested after to EM164 exposure at given time points. Protein lysates were resolved by electrophoresis on 7% SDS–PAGE, transferred to nitrocellulose membrane and visualised by Western blotting with specific antibodies, detected with ECL plus reagent. The blots were reprobed with anti-actin antibody as loading control. IGF-I stimulation was used as control, but not given during EM164 exposure.

($r = 0.6167$, $p = 0.0857$, non-parametric two-tailed Spearman; Fig. 1B).

3.3. *EM164 inhibits IGF-1R, AKT and mitogen-activated protein kinase (MAPK) phosphorylation in neuroblastoma cells*

IMR32, SH-SY5Y and SH-N-SH cells exhibited activated IGF-1R, which could be further stimulated by IGF-I (Fig. 2). EM164 at 0.7 μg/mL resulted in the complete inhibition of constitutive IGF-1R phosphorylation during at least 48 h in all three cell lines while total expression of the IGF-1R was not modified. Inhibition of MAPK and AKT phosphorylation was noted in all three cell lines, although this did not result in caspase-3 activation or PARP cleavage (data not shown). Thus, EM164 reduced cell proliferation through inhibition of IGF-1R-mediated phosphatidyl inositol 3 kinase (PI3K)/AKT and MAPK cell signalling pathways but not through induction of apoptotic cell death.

3.4. *EM164 induces G1 cell arrest in neuroblastoma cells*

EM164 treatment resulted in G1 cell cycle arrest in SK-N-AS and IGR-N91 cells. For IGR-N91, 60% of EM164-treated cells were in G0/1 phase at 24 h compared to 40% of control cells and 62% versus 51% at 48 h, respectively. For SK-N-AS, at 24 h 76% of EM164-treated cells were in G0/1 compared to 64% in the controls and at 48 h 63% versus 53%, respectively. At 72 h, the cell cycle distribution was similar in EM164-treated and control cells (54% versus 54% for IGR-N91, 77% and 72% for SK-N-AS). The increase of cells in G1 fraction was to the disadvantage of cells in S-phase and no enhanced sub-G1 fraction was observed. Thus, EM164 exhibits anti-proliferative activity through G1 cell cycle arrest.

3.5. *EM164 has superior activity with temozolomide in neuroblastoma cells*

We evaluated anti-proliferative effects of EM164 combined with temozolomide (Fig. 3A). Both agents were first administered simultaneously, EM164 at 0.7 μg/mL and temozolomide at two doses which can induce 40–60% viability inhibition in each cell line. All combinations exhibited superior anti-proliferative activity. In IGR-N91, EM164 treatment resulted in 68% (±4%, 95% confidence interval (CI)) cell viability compared to untreated controls, temozolomide at 800 μM in 55% (±3%), while the combined treatment resulted in 28% (±3%) cell viability. Similarly, temozolomide at 400 μM resulted in 68% (±7%) viability, the combination with EM164 in 44% (±3%). In SK-N-AS cells, cell viability was 76% (±6%) following EM164 treatment, 61% (±4%) for temozolomide at 200 μM and 42% (±3%) for the EM164-temozolomide combination. Temozolomide at 400 μM resulted in 51% (±5%) cell viability and its combination with EM164 in 35% (±3%). We also evaluated the sequencing of EM164 and temozolomide treatments. Anti-proliferative effects were equivalent when EM164 was given 48 h prior to or 48 h following temozolomide. Thus, EM164 and temozolomide exhibit enhanced anti-proliferative effects *in vitro* which appear independent of the sequencing of both treatments.

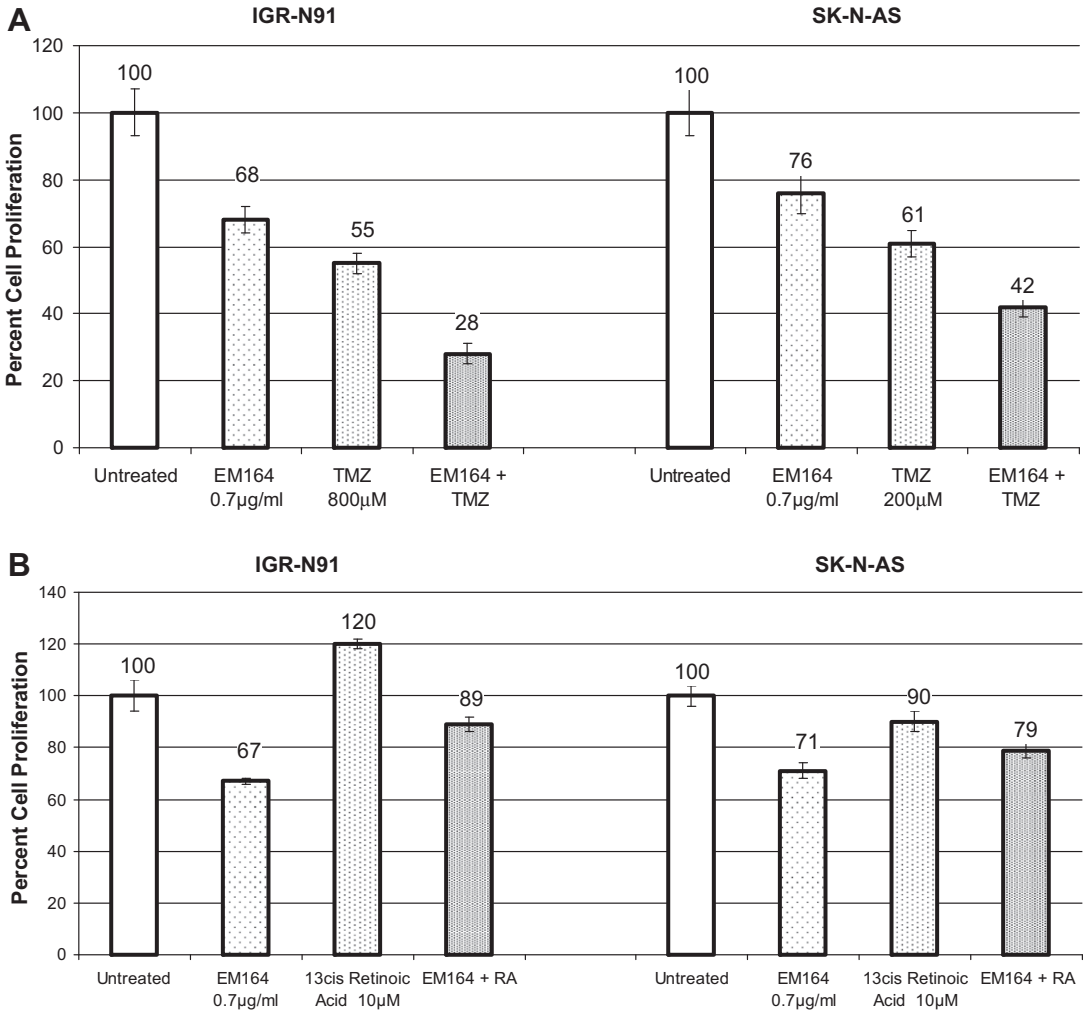

Fig. 3 – Anti-proliferative activity of EM164 in combination with temozolomide (A) or cis-retinoid acid (B) in IGR-N91 and SK-N-AS cell lines. Cell proliferation of IGR-N91 and SK-N-AS cells cultured in 10% foetal bovine serum-conditioned medium after 48 h exposure to EM164 at 0.7 μg/mL and temozolomide (A) or 13 cis-RA (B) at indicated concentrations, as measured by MTT assay. Data represent means out of at least three independent experiments each performed in triplicates.

3.6. *Cis-retinoic acid reduces anti-proliferative activity of EM164 in neuroblastoma cells*

We next evaluated the combination of EM164 with cis-retinoid acid (cis-RA) (Fig. 3B). Similar to the prior combination experiments, EM164 reduced cell viability of SK-N-AS cells to 71% (±3% 95% CI) and of IGR-N91 to 67% (±1%). While cis-RA exhibited a moderate anti-proliferative effect in SK-N-AS cells (90% (±4%) viability), this differentiating agent stimulated IGR-N91 proliferation (120% (±2%)). Importantly, when administered simultaneously, cell viability inhibition of EM164 was reduced in both cell lines (79% (±3%) in SK-N-AS and 89% (±3%) in IGR-N91). Sequencing experiments of the combination in SK-N-AS showed comparable viability inhibition when EM164 was administered 48 h prior to or 48 h after cis-RA with cell viability rates of 62% versus 62%, respectively. In contrast in RA-resistant IGR-N91 cells, EM164 exposure during 48 h prior to cis-RA resulted in 83% cell viability, while no viability inhibition (110% viability) was observed when EM164 was administered after 48 h cis-RA exposure. EM164, when given prior to RA, may have the ability to partially reverse resistance to RA in IGR-N91 cells. In summary, concomitant exposure of cis-RA reduced anti-proliferative effects of EM164 *in vitro*.

3.7. *EM164 exhibits significant anti-tumour activity in IGR-N91 and SK-N-AS xenografts*

EM164 was evaluated *in vivo* against advanced stage xenografts. Treatment started 23 and 18 d after subcutaneous transplantation of IGR-N91 and SK-N-AS tumours, respectively, at 40 mg/kg twice weekly for 28 d and 24 d, i.e. nine and eight injections, respectively (Fig. 4; Table 1). Vehicle-treated IGR-N91 tumours grew with a median time to reach 750 mm^3 of 10.5 days, EM164-treated tumours of 23.9 days, indicating a significant growth delay *T–C* of 13.4 days ($p = 0.02$; Mann–Whitney). One EM164-treated tumour regressed below <63 mm^3, regrew slowly and had not reached 750 mm^3 as of study end, on day 39. With a total log cell kill of 0.86, EM164 was declared active according to the SRI score.

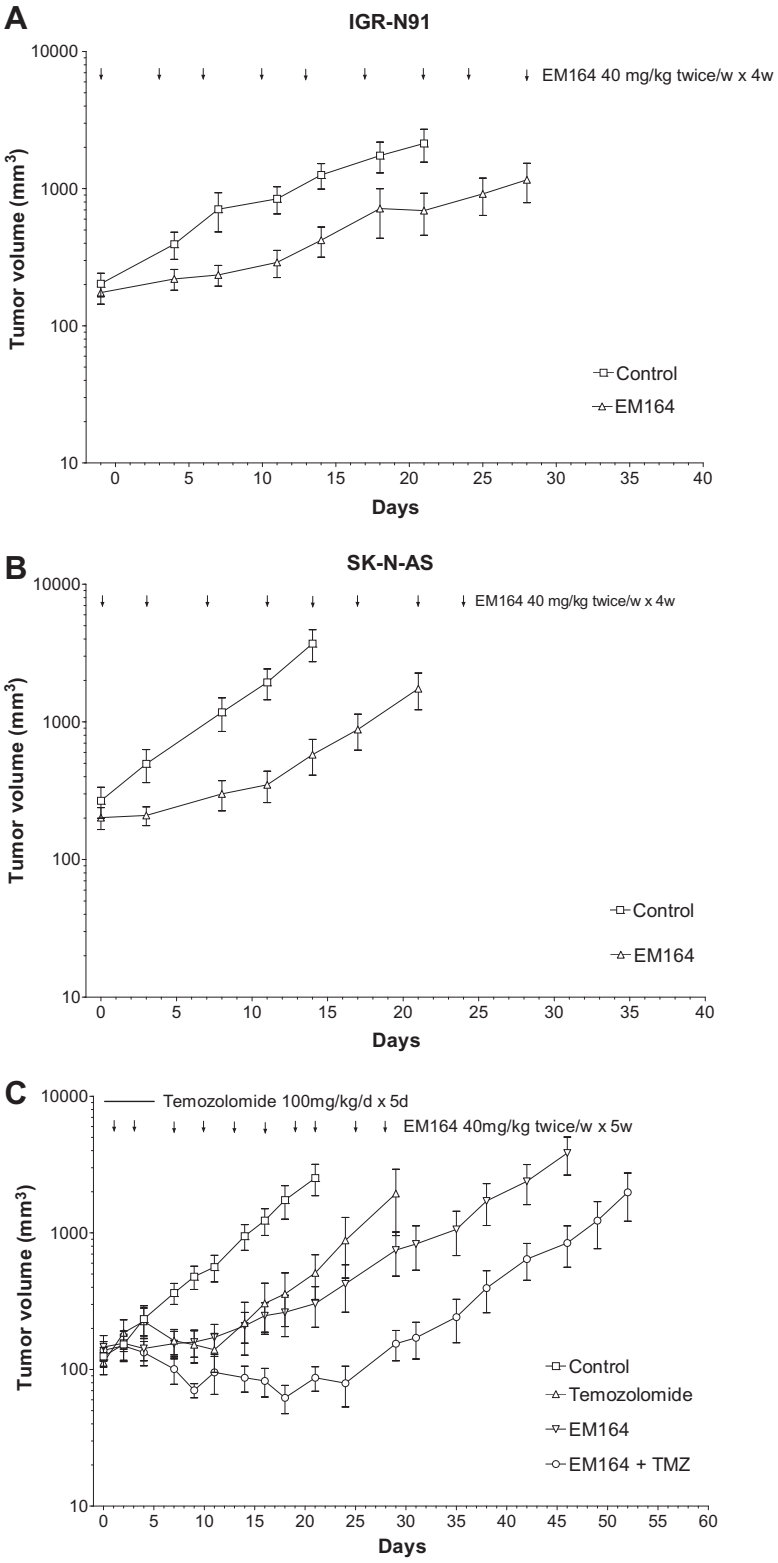

Fig. 4 – Anti-tumour activity of EM164 in advanced neuroblastoma xenografts IGR-N91 (A) and SK-N-AS (B) and in combination with temozolomide in SK-N-AS (C). Animals bearing advanced IGR-N91 or SK-N-AS tumours were assigned to treatment with vehicle and/or Klucel 0.2 mL, EM164 at 40 mg/kg twice weekly, and/or temozolomide 100 mg/kg during five consecutive days. Graphs are presented with the mean tumour lead for each treatment group of 5–8 animals; bars are standard error of means.

Table 1 – Anti-tumour activity of EM164 alone and with temozolomide in neuroblastoma xenografts.

	Treatment	Total dose (mg/kg)	Mice (n)	Tumour load median (mm^3) (range)	Td (days)	Weight loss (%) (D29)	CR	T 750 mm^3 (days)	T–C (days)	*p*	LCK (SRI criteria)
EM164 against IGR-N91											
Vehicle	0.2 mL × 2/w × 28 d		8	183 (75–408)	4.7	+4 (D24)	0	10.5			
EM164	40 mg/kg × 2/w × 28 d	320	8	166 (80–348)		+6 (D28)	1	23.9	13.4	0.02	0.86 (+)
EM164 against SK-N-AS											
Vehicle	0.2 mL × 2/w × 24 d		8	175 (76–536)	3.0	+9 (D17)	0	6.3			
EM164	40 mg/kg × 2/w × 24 d	280	7	191 (85–362)		+11 (D21)	0	19.2	12.9	0.0059	1.30 (++)
EM164 and temozomide against SK-N-AS											
Vehicle	Klucel 0.2 mL × 5 d PBS 0.2 mL × 2/w × 5 w		5	135 (58–183)	3.4	+12 (D24)	0	11.7			
EM164	40 mg/kg × 2/w × 5 w	400	5	150 (72–224)		+4	1	29.8	18.1	ns	1.6 (++)
Temozolomide	100 mg/kg × 5 d	500	5	127 (66–168)		+9	0	27.8	16.1	ns	1.4 (++)
Temozolomide + EM164[a]	100 mg/kg × 5d + 40 mg/kg × 2/w × 5 w	500 400	5	170 (80–177)		+3	2	40.8	29.1	<0.01	2.6 (+++)

Td = tumour doubling time; CR = complete regression; 5 × Vi = five times initial tumour volume; T 750 mm^3 = median time to reach 750 mm^3; *T–C* = tumour growth delay; *p* = *T–C* statistical difference using a two-tailed non-parametric Mann–Whitney or Kruskal–Wallis test for two or more groups, respectively; LCK = log cell kill. Southern Research Institute (SRI) criteria: highly active ++++ = log cell kill gross (treatment duration of 5–20 d) > 2.8, +++ = 2.0–2.8, ++ = 1.3–1.9, + = 0.7–1.2, inactive − ⩽ 0.7.

[a] The first EM164 was administered after the second temozolomide dosing.

Median time of vehicle-treated SK-N-AS tumours to reach 750 mm^3 was 6.3 days, that of EM164-treated tumours was 19.2 days. Thus with a significant *T–C* of 12.9 days ($p = 0.0059$), EM164 was also found to be active against SK-N-AS neuroblastoma (1.3 total log cell kill).

3.8. EM164 anti-tumour activity correlates with AKT inhibition and cell death in SK-N-AS xenografts

Morphological and pharmacodynamic changes in SK-N-AS tumours were analysed 24 h after the second EM164 administration. Control tumours had doubled their mean volume (253 mm^3 on day 4 versus 121 mm^3 on day 0) while EM164-treated tumours remained stable or regressed (mean: 95 mm^3 versus 105 mm^3). Post HES staining, untreated SK-N-AS tumours presented undifferentiated neuroblasts with large hyperchromatic nuclei and multiple cell mitosis (Fig. 5, panel A). EM164-treated tumours presented important post-therapeutic changes with foci of pre-necrotic tumour cells showing pyknotic and dystrophic nuclei, marked chromatin condensation, surrounded by enhanced fibrotic tissue (panel B–C), which was particular evident in regressing tumours. Consistent with the *in vitro* findings, immunohistochemistry did not suggest induction of caspase-3-dependent apoptotic cell death (data not shown). In contrast, p-AKT expression was significantly reduced in EM164-treated tumours as shown by immunohistochemistry (panels D–F) and Western blot analysis in tumour lysates (55% decrease of the levels of phosphorylation of AKT on serine 473 ($p = 0.0159$) as compared to control tumours, the levels of total AKT being not modified). Thus, inhibition of AKT activation appears to be a predictive marker for EM164 efficacy *in vivo*.

3.9. EM164 and temozolomide exhibit superior anti-tumour activity in SK-N-AS xenografts

Finally, we explored *in vivo* EM164 in combination with temozolomide in SK-N-AS (Fig. 4C; Table 1). Due to the EM164-induced G1 cell growth arrest, temozolomide for five days was given at days 0–4 and EM164 injections twice weekly during 32 days, i.e. 10 injections, starting on day 1 after the second temozolomide dosing. EM164 was declared active based on SRI score with 1.6 log cell kill but no statistical significance was achieved in terms of TGD compared to vehicle-treated tu-

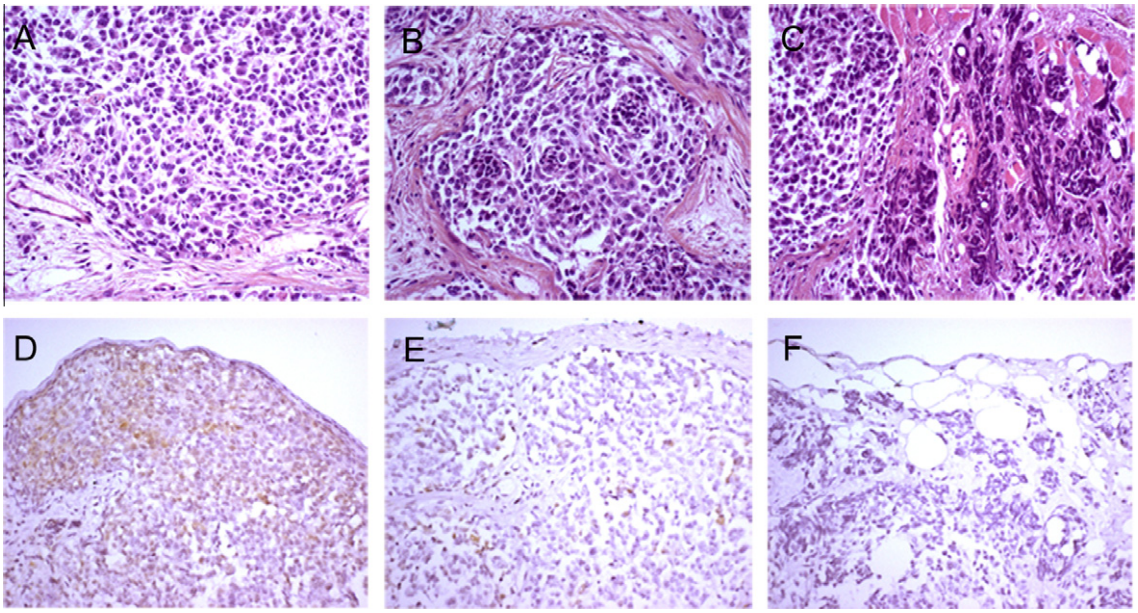

Fig. 5 – HES staining and p-AKT expression of SK-N-AS xenograft tumours treated with EM164. Panels A–C show SK-N-AS tumour morphology in HES staining of untreated controls (A) and post-therapeutic changes (i.e. fibrosis and pyknotic nuclei) 24 h after two EM164 administrations (B and C). Panels D–F show p-AKT expression in control tumours (D) and inhibition of AKT phosphorylation after the second EM164 administration (E and F; 200× original magnification).

mours (*T–C* = 18.1 days; p = ns using Kruskal–Wallis test). One EM164-treated animal experienced complete regression with slow-regrowing tumours. Temozolomide at the maximum tolerated dose of 100 mg/kg/d for 5 d (as defined based on previous studies in our laboratory) achieved a tumour growth delay *T–C* of 16.1 d (p = ns) without tumour regression and a total log cell kill of 1.4. Combination of temozolomide with EM164 yielded a significant tumour growth delay (*T–C* = 29.1 d; $p < 0.01$) with a greater total log cell kill of 2.6 as compared to the single agents. Two animals experienced complete tumour regression. In conclusion, combination of EM164 with temozolomide results in enhanced therapeutic activity.

4. Discussion

This is the first study reporting significant anticancer activity of a monoclonal antibody currently in clinical development in childhood neuroblastoma, as single agent and in combination with alkylating agents. EM164 (the murine AVE1642) exhibited anti-proliferative activity in neuroblastoma cells at the lowest concentration tested (0.07 μg/mL) which compares favourably to the 10 μg/mL used in other cancers (Bladt et al., unpublished data).[7,24] Similarly, activity was observed under serum and serum-reduced growth conditions without supplementary IGF-I stimulations. Neuroblastoma cell sensitivity was independent from IGF-1R levels and IGF-II secretion (data not shown), but associated with inhibition of IGF-1R phosphorylation, PI3K/AKT and MAPK cell signalling. This is consistent with findings reported for the h10H5 antibody[25] and the NVP-AEW541 small molecule.[20,26] Moreover, inhibition of AKT phosphorylation appears to correlate with tumour regression *in vivo*; this could represent a biomarker for this treatment, but needs to be confirmed.

EM164 exerts its main activity through G1 cell cycle arrest, while we found no evidence for the induced apoptotic cell death. In all three cell lines tested, IGF-1R phosphorylation was inhibited without down-regulation of the total IGF-1R as described previously.[7] Receptor down-regulation due to receptor internalisation via clathrin-mediated endocytosis and endosomal and lysosomal degradation is primarily observed following antibody treatment rather than tyrosine kinase inhibitors,[27] and considered to be a prerequisite for the induction of apoptosis.[28] Despite the absence of caspase-3 expression *in vivo*, SK-N-AS tumours regressing under EM164 treatment showed morphological signs of cell death, i.e. pyknotic nuclei, chromatin condensation and fibrous tissue replacement which suggest either caspase-independent apoptotic cell death or other cell death processes being involved. In prior studies, Descamps et al. also found no EM164-induced apoptosis *in vitro*,[24] while enhanced TUNEL-positive cells were reported for the IMC-A12 antibody[29] and the tyrosine kinase inhibitors NVP-AEW541 and BMS-536924.[26,30] Clearly, further information is needed to understand the mechanism of action of these types of agents.

All nine representative neuroblastoma cell lines and both xenograft models tested were sensitive to EM164. IGF-1R promoter activity is reported to be enhanced by *MYCN* and *MYCC*[15] and suppressed by wild-type *TP53*, *BRCA1* and *WT1*,[31–33] suggesting *MYCN*-amplified neuroblastoma with activated IGF-1R pathway. Importantly, sensitivity to EM164 was independent from MYCN status. Neuroblastoma is a heterogeneous tumour with more tumourigenic motile neuronal-type cells, reported to express higher levels of IGF-1R, and less tumourigenic, more adherent stromal-type cells.[34] We did not share these findings and found for example higher IGF-1R levels in the stromal-type SK-N-AS cells as compared

to the neuronal SH-SY5Y and IMR32; moreover, EM164 exhibited activity in both types.

Both xenografts subsequently escaped EM164 treatment. IGF-1R inhibition escape mechanisms are currently a 'hot topic'. The IGF-1R pathway is involved in a complex signalling cross-talk through multiple growth factors, receptors and down-stream effectors. At present it is not clear whether and to what extend the IGF axis is essential for the maintenance of the malignant phenotype. Activating *IGF-1R* gene mutations and amplification are rare,[1] while polymorphisms or epigenetic changes in IGFBPs, IGF-I and IGF-II, due to methylation or loss of imprinting at the *IGF-II* gene locus,[35] as well as factors that activate PI3K/AKT pathway, i.e. phosphatase and tensin homologue (PTEN) loss, *PIK3CA*-activating mutations, or expression of Heat Shock Protein (HSP) 90, are described. Alternative signalling of IGF-I and IGF-II through other insulin-related receptors, like the IR,[36] or hetero-dimerisation of IGF-1R with other receptors, i.e. IR, EGFR (epidermal growth factor receptor), ERBB2 or platelet-derived growth factor receptor (PDGFR), may bypass therapeutic antibodies to IGF-1R that have primarily no inhibitory activity to the other receptors, as is the case for tyrosine kinase inhibitors for IR.[21,30,37,38] As IGF-1R upregulation mediates resistance to EGFR, HER2 or IR inhibitors,[21,39,40] it is possible that reciprocal upregulation of EGFR family members, IR or alternative signalling pathways negate IGF-1R targeting effects and overexpression of IGFBP-3, IGFBP-6, EGFR and its ligands was found associated with resistance to BMS-536924 in sarcoma and neuroblastoma cell lines.[30] Preclinical studies showed superior activity when IGF-1R targeting was combined with those of HER2,[41] EGFR,[42,43] mammalian target of rapamycin (mTOR),[16] HSP90 or PI3K;[44] several of these combinations are being evaluated in clinical trials.

Another approach to improve targeted agents has been their combination with cytotoxic agents. We have shown enhanced activity of EM164 with the alkylating agent temozolomide recently proven active in children with relapsed neuroblastoma.[45] *In vivo*, temozolomide treatment was started before EM164 while their sequencing appeared irrelevant for enhanced effects *in vitro*. Nevertheless, cell cycle-dependent anticancer agents may need to be administered prior to the antibody. Enhanced activity of IGF-1R inhibition has also been reported with doxorubicin, 5-fluorouracil, tamoxifen,[42] docetaxel,[46] vincristine, actinomycin D and ifosphamide, while sub-additive effects were observed with doxorubicin and cisplatin and NVP-AEW541.[26] Dallas et al. suggested a potential interest of IGF-1R targeting in chemoresistant cancers which often exhibit a high percentage of cancer stem cells.[47] Ongoing clinical phase Ib studies associate IGF-1R antibodies with docetaxel (AVE1642), carboplatin and paclitaxel (CP751–871, NCT00147537). We further explored whether IGF-1R inhibition may be favourably added in the current high-risk neuroblastoma protocol using retinoic acid. Neurite outgrowth is regulated by IGFs,[48] while constitutive IGF-II expression was suggested to be implicated in RA-resistance.[49,50] As described previously, we found neuroblastoma cells that may arrest and differentiate (SK-N-AS) as well as those termed resistant to RA (IGR-N91). Indeed we found the RA-resistant IGR-N91 cells more sensitive when EM164 was added. However, in both cell lines tested, concomitant cis-RA treatment reduced the anti-proliferative capacity of EM164 suggesting that it should not be associated during maintenance therapy.

In conclusion, significant anti-proliferative and anti-tumour activity in all tested advanced neuroblastoma models and enhanced effects with alkylating agents demonstrate the potential of the AVE1642 antibody and support its development for neuroblastoma. To date predictive markers of IGF-1R treatment sensitivity are unknown and pharmacodynamic read-outs may help to define sensitive or resistant tumours to this promising treatment approach.

Conflict of interest statement

Corinne Venot, Laurent Debussche and Patricia Vrignaud are employees of sanofi-aventis 94493 Vitry/Seine CEDEX, France. The other authors have no conflict of interest to declare.

Acknowledgements

We are grateful to Dr. Patrick Gonin and his team at the animal facility of IGR, to Ms. Olivia Bawas for her excellent technical assistance of histology, Mr. Yann Lecluse for flow cytometry analysis and thank Dr. Cormac Owens for critical reading of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.06.005.

REFERENCES

1. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. *Nat Rev Cancer* 2004;**4**(7):505–18.
2. Baserga R, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. *Int J Cancer* 2003;**107**(6):873–7.
3. Barlaskar FM, Spalding AC, Heaton JH, et al. Preclinical targeting of the type I insulin-like growth factor receptor in adrenocortical carcinoma. *J Clin Endocrinol Metab* 2009;**94**(1):204–12.
4. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999;**13**(22):2905–27.
5. Zhang H, Yee D. The therapeutic potential of agents targeting the type I insulin-like growth factor receptor. *Expert Opin Invest Drugs* 2004;**13**(12):1569–77.
6. Schumacher R, Soos MA, Schlessinger J, et al. Signaling-competent receptor chimeras allow mapping of major insulin receptor binding domain determinants. *J Biol Chem* 1993;**268**(2):1087–94.
7. Maloney EK, McLaughlin JL, Dagdigian NE, et al. An anti-insulin-like growth factor I receptor antibody that is a potent inhibitor of cancer cell proliferation. *Cancer Res* 2003;**63**(16):5073–83.
8. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 1993;**75**(1):59–72.

9. El-Badry OM, Romanus JA, Helman LJ, et al. Autonomous growth of a human neuroblastoma cell line is mediated by insulin-like growth factor II. *J Clin Invest* 1989;**84**(3):829–39.
10. Castle VP, Heidelberger KP, Bromberg J, et al. Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. *Am J Pathol* 1993;**143**(6):1543–50.
11. Puglianiello A, Germani D, Rossi P, Cianfarani S. IGF-I stimulates chemotaxis of human neuroblasts. Involvement of type 1 IGF receptor, IGF binding proteins, phosphatidylinositol-3 kinase pathway and plasmin system. *J Endocrinol* 2000;**165**(1):123–31.
12. van Golen CM, Schwab TS, Kim B, et al. Insulin-like growth factor-I receptor expression regulates neuroblastoma metastasis to bone. *Cancer Res* 2006;**66**(13):6570–8.
13. Weber A, Huesken C, Bergmann E, et al. Coexpression of insulin receptor-related receptor and insulin-like growth factor 1 receptor correlates with enhanced apoptosis and dedifferentiation in human neuroblastomas. *Clin Cancer Res* 2003;**9**(15):5683–92.
14. van Golen CM, Castle VP, Feldman EL. IGF-I receptor activation and BCL-2 overexpression prevent early apoptotic events in human neuroblastoma. *Cell Death Differ* 2000;**7**(7):654–65.
15. Chambery D, Mohseni-Zadeh S, de Galle B, Babajko S. N-myc regulation of type I insulin-like growth factor receptor in a human neuroblastoma cell line. *Cancer Res* 1999;**59**(12):2898–902.
16. Coulter DW, Blatt J, D'Ercole AJ, Moats-Staats BM. IGF-I receptor inhibition combined with rapamycin or temsirolimus inhibits neuroblastoma cell growth. *Anticancer Res* 2008;**28**(3A):1509–16.
17. Liu X, Turbyville T, Fritz A, Whitesell L. Inhibition of insulin-like growth factor I receptor expression in neuroblastoma cells induces the regression of established tumors in mice. *Cancer Res* 1998;**58**(23):5432–8.
18. Meyer GE, Chesler L, Liu D, et al. Nordihydroguaiaretic acid inhibits insulin-like growth factor signaling, growth, and survival in human neuroblastoma cells. *J Cell Biochem* 2007;**102**(6):1529–41.
19. Coulter DW, Wilkie MB, Moats-Staats BM. Inhibition of IGF-I receptor signaling in combination with rapamycin or temsirolimus increases MYC-N phosphorylation. *Anticancer Res* 2009;**29**(6):1943–9.
20. Tanno B, Mancini C, Vitali R, et al. Down-regulation of insulin-like growth factor I receptor activity by NVP-AEW541 has an antitumor effect on neuroblastoma cells in vitro and in vivo. *Clin Cancer Res* 2006;**12**(22):6772–80.
21. Haluska P, Carboni JM, Loegering DA, et al. In vitro and in vivo antitumor effects of the dual insulin-like growth factor-I/insulin receptor inhibitor, BMS-554417. *Cancer Res* 2006;**66**(1):362–71.
22. Vassal G, Terrier-Lacombe MJ, Bissery MC, et al. Therapeutic activity of CPT-11, a DNA-topoisomerase I inhibitor, against peripheral primitive neuroectodermal tumour and neuroblastoma xenografts. *Brit J Cancer* 1996;**74**(4):537–45.
23. Ferrandis E, Da Silva J, Riou G, Benard I. Coactivation of the MDR1 and MYCN genes in human neuroblastoma cells during the metastatic process in the nude mouse. *Cancer Res* 1994;**54**(8):2256–61.
24. Descamps G, Gomez-Bougie P, Venot C, et al. A humanised anti-IGF-1R monoclonal antibody (AVE1642) enhances Bortezomib-induced apoptosis in myeloma cells lacking CD45. *Brit J Cancer* 2009;**100**(2):366–9.
25. Shang Y, Mao Y, Batson J, et al. Antixenograft tumor activity of a humanized anti-insulin-like growth factor-I receptor monoclonal antibody is associated with decreased AKT activation and glucose uptake. *Mol Cancer Ther* 2008;**7**(9):2599–608.
26. Scotlandi K, Manara MC, Nicoletti G, et al. Antitumor activity of the insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 in musculoskeletal tumors. *Cancer Res* 2005;**65**(9):3868–76.
27. Manara MC, Landuzzi L, Nanni P, et al. Preclinical in vivo study of new insulin-like growth factor-I receptor – specific inhibitor in Ewing's sarcoma. *Clin Cancer Res* 2007;**13**(4):1322–30.
28. Baserga R. The insulin-like growth factor-I receptor as a target for cancer therapy. *Expert Opin Ther Targets* 2005;**9**(4):753–68.
29. Burtrum D, Zhu Z, Lu D, et al. A fully human monoclonal antibody to the insulin-like growth factor I receptor blocks ligand-dependent signaling and inhibits human tumor growth in vivo. *Cancer Res* 2003;**63**(24):8912–21.
30. Huang F, Greer A, Hurlburt W, et al. The mechanisms of differential sensitivity to an insulin-like growth factor-1 receptor inhibitor (BMS-536924) and rationale for combining with EGFR/HER2 inhibitors. *Cancer Res* 2009;**69**(1):161–70.
31. Werner H, Karnieli E, Rauscher FJ, LeRoith D. Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. *Proc Natl Acad Sci USA* 1996;**93**(16):8318–23.
32. Werner H, Roberts Jr CT, Rauscher III FJ, LeRoith D. Regulation of insulin-like growth factor I receptor gene expression by the Wilms' tumor suppressor WT1. *J Mol Neurosci* 1996;**7**(2):111–23.
33. Abramovitch S, Glaser T, Ouchi T, Werner H. BRCA1–Sp1 interactions in transcriptional regulation of the IGF-IR gene. *FEBS Lett* 2003;**541**(1–3):149–54.
34. Kim B, van Golen CM, Feldman EL. Insulin-like growth factor-I signaling in human neuroblastoma cells. *Oncogene* 2004;**23**(1):130–41.
35. Feinberg AP. The epigenetics of cancer etiology. *Semin Cancer Biol* 2004;**14**(6):427–32.
36. Ullrich A, Gray A, Tam AW, et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* 1986;**5**(10):2503–12.
37. Sachdev D, Singh R, Fujita-Yamaguchi Y, Yee D. Down-regulation of insulin receptor by antibodies against the type I insulin-like growth factor receptor: implications for anti-insulin-like growth factor therapy in breast cancer. *Cancer Res* 2006;**66**(4):2391–402.
38. Ji QS, Mulvihill MJ, Rosenfeld-Franklin M, et al. A novel, potent, and selective insulin-like growth factor-I receptor kinase inhibitor blocks insulin-like growth factor-I receptor signaling in vitro and inhibits insulin-like growth factor-I receptor dependent tumor growth in vivo. *Mol Cancer Ther* 2007;**6**(8):2158–67.
39. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst* 2001;**93**(24):1852–7.
40. Chakravarti A, Loeffler JS, Dyson NJ. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. *Cancer Res* 2002;**62**(1):200–7.
41. Camirand A, Lu Y, Pollak M. Co-targeting HER2/ErbB2 and insulin-like growth factor-1 receptors causes synergistic inhibition of growth in HER2-overexpressing breast cancer cells. *Med Sci Monit* 2002;**8**(12):BR521–6.
42. Cohen BD, Baker DA, Soderstrom C, et al. Combination therapy enhances the inhibition of tumor growth with the fully human anti-type 1 insulin-like growth factor receptor

monoclonal antibody CP-751, 871. *Clin Cancer Res* 2005;**11**(5):2063–73.
43. Lu D, Zhang H, Koo H, et al. A fully human recombinant IgG-like bispecific antibody to both the epidermal growth factor receptor and the insulin-like growth factor receptor for enhanced antitumor activity. *J Biol Chem* 2005;**280**(20):19665–72.
44. Martins AS, Ordonez JL, Garcia-Sanchez A, et al. A pivotal role for heat shock protein 90 in Ewing sarcoma resistance to anti-insulin-like growth factor 1 receptor treatment: in vitro and in vivo study. *Cancer Res* 2008;**68**(15):6260–70.
45. Rubie H, Chisholm J, D7efachelles AS, et al. Phase II study of temozolomide in relapsed or refractory high-risk neuroblastoma: a joint Societe Francaise des Cancers de l'Enfant and United Kingdom Children Cancer Study Group-New Agents Group Study. *J Clin Oncol* 2006;**24**(33):5259–64.
46. Rowinsky EK, Youssoufian H, Tonra JR, et al. IMC-A12, a human IgG1 monoclonal antibody to the insulin-like growth factor I receptor. *Clin Cancer Res* 2007;**13**(18 Pt 2): 5549s–55s.
47. Dallas NA, Xia L, Fan F, et al. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. *Cancer Res* 2009;**69**(5):1951–7.
48. Babajko S, Binoux M. Modulation by retinoic acid of insulin-like growth factor (IGF) and IGF binding protein expression in human SK-N-SH neuroblastoma cells. *Eur J Endocrinol* 1996;**134**(4):474–80.
49. Matsumoto K, Lucarelli E, Minniti C, Gaetano C, Thiele CJ. Signals transduced via insulin-like growth factor I receptor (IGF(R)) mediate resistance to retinoic acid-induced cell growth arrest in a human neuroblastoma cell line. *Cell Death Differ* 1994;**1**(1):49–58.
50. Gaetano C, Matsumoto K, Thiele CJ. Retinoic acid resistant neuroblastoma cells and the expression of insulin-like growth factor-II. *Prog Clin Biol Res* 1991;**366**:165–72.