European Journal of Cancer (2014) 50, 2463–2477

Available at www.sciencedirect.com

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OTX008, a selective small-molecule inhibitor of galectin-1, downregulates cancer cell proliferation, invasion and tumour angiogenesis

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Received 11 February 2014; received in revised form 11 June 2014; accepted 16 June 2014
Available online 16 July 2014

KEYWORDS
Galectin-1
Calixarene compounds
Anginex
Cell proliferation
Cell invasion
Neuropilin-1

Abstract ***Background:*** Galectin-1 (Gal1), a carbohydrate-binding protein is implicated in cancer cell proliferation, invasion and tumour angiogenesis. Several Gal1-targeting compounds have recently emerged. OTX008 is a calixarene derivative designed to bind the Gal1 amphipathic β-sheet conformation. Our study contributes to the current understanding of the role of Gal1 in cancer progression, providing mechanistic insights into the anti-tumoural activity of a novel small molecule Gal1-inhibitor.
Methods: We evaluated *in vitro* OTX008 effects in a panel of human cancer cell lines. For *in vivo* studies, an ovarian xenograft model was employed to analyse the antitumour activity. Finally, combination studies were performed to analyse potential synergistic effects of OTX008.
Results: In cultured cancer cells, OTX008 inhibited proliferation and invasion at micromolar concentrations. Antiproliferative effects correlated with Gal1 expression across a large panel of cell lines. Furthermore, cell lines expressing epithelial differentiation markers were more

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http://dx.doi.org/10.1016/j.ejca.2014.06.015

sensitive than mesenchymal cells to OTX008. In SQ20B and A2780-1A9 cells, OTX008 inhibited Gal1 expression and ERK1/2 and AKT-dependent survival pathways, and induced G2/M cell cycle arrest through CDK1. OTX008 enhanced the antiproliferative effects of Semaphorin-3A (Sema3A) in SQ20B cells and reversed invasion induced by exogenous Gal1. *In vivo*, OTX008 inhibited growth of A2780-1A9 xenografts. OTX008 treatment was associated with downregulation of Gal1 and Ki67 in treated tumours, as well as decreased microvessel density and VEGFR2 expression. Finally, combination studies showed OTX008 synergy with several cytotoxic and targeted therapies, principally when OTX008 was administered first.

Conclusion: This study provides insights into the role of Gal1 in cancer progression as well as OTX008 mechanism of action, and supports its further development as an anticancer agent.

1. Introduction

Galectins are carbohydrate-binding lectins, defined by their affinity for β-galactoside-containing glycans [1]. The galectin family is defined by a consensus amino acid sequence and the presence of at least one conserved carbohydrate-recognition domain (CRD) which is responsible for its binding to N- and O-linked glycans [2]. Galectin family members are classified in three groups according to the number and structure of the CRD domains: galectins bearing one CRD or two homologous CRDs connected by a short linker peptide and galectin-3, the only galectin with one CRD fused to tandem repeats of short amino-acid stretches [1].

Galectin-1 (Gal1) carrying one CRD, plays multiple roles in several physiologic and pathologic processes [3,4]. In tumour progression, it is involved in cell–cell and cell-extracellular matrix (ECM) interactions [5,6], proliferation [6,7], invasion [6,8], angiogenesis [6,9], and escape from immune surveillance [6,10]. Gal1 is overexpressed in tumour cells and tumour-associated endothelial cells [6,11]. Upregulation has been linked with poor clinical prognosis and metastases development in a wide range of malignancies including gastric [12], breast [10], ovarian [13], prostate [14], colorectal [15] and head & neck (H&N) squamous cell [16] carcinomas glioblastoma [17] and Kaposi's sarcoma [18].

Intracellular Gal1 can enhance cell proliferation and tumour transformation by protein–protein interactions with oncogenic RAS [19] or with the nuclear protein Gemin4 induced in RNA splicing [20]. The role of Gal1 in tumour cell signalling via activation of the mitogen activated proliferation kinase (MAPK) and other intracellular survival cascades has been well documented [6,21]. Extracellular Gal1 dimerises and forms multivalent bonds with a wide array of glycoproteins and glycolipids on the cell surface, as well as with ECM components such as fibronectin and laminin, activating intracellular signalling pathways which modulate cell proliferation and promote epithelial-to-mesenchymal transition (EMT) [6,22,23]. In addition, evidence that Gal1 promotes EMT has been reported in a lung cancer cell line [24]. Furthermore, binding of Gal1 to cell surface and ECM enhances tumour cell migration and invasion as well as dispersion of metastases [6,25]. In addition, its secretion by tumour cells enhances endothelial cell activity and neovessel formation revealing a role in tumour angiogenesis [9,14,26]. Neuropilin-1 (NRP-1) is a type I transmembrane glycoprotein found in mesenchymal stem cells and tumour-associated stromal and endothelial cells [27,28]. Binding to its principal ligand Semaphorin-3A (Sema3A) inhibits proliferation and metastasis of breast carcinoma and melanoma [29–31]. This interaction blocks endothelial cell migration and initiates antiangiogenic signalling cascades, mainly through its interaction with Plexin-A [32]. It was shown that Gal1 selectively binds to NRP-1 via its CRD domain, activating VEGFR2 signalling cascades, resulting in increased proliferation, migration and adhesion of endothelial cells [33].

Recent advances in understanding Gal1 functions in cancer have presented opportunities to develop novel targeting strategies [6]. Different approaches to inhibit this lectin are currently under investigation such as blocking CRD using oligosaccharides and derivatives or specific monoclonal antibodies [6]. These compounds showed interesting effects on tumour cell proliferation, invasion and angiogenesis, however pharmacokinetic and pharmacodynamic limitations and poor Gal1 selectivity have restricted the development of several of these novel leads [6]. A series of synthetic peptides were designed based on the 3-dimensional β-sheet structures of the β-chemokines platelet factor 4 and interleukin-8 [34,35]. The most potent amongst them was anginex, a 33-amino acid peptide, which specifically interacts with and inhibits Gal1 functions [9], as well as decreases tumour growth and angiogenesis in several tumour models [36]. To improve the pharmacokinetics of this agent, non-peptidic compounds were designed with comparable molecular dimensions, hydrophobic and positively-charged amino acid composition and similar surface topology of the functionally β-sheet conformation of anginex [37]. We recently showed that OTX008 (0118, PTX-008; Fig. 1), a novel calixarene compound derived from anginex, binds to Gal1 on the side back face, away from the β-galactoside-binding site [38].

Previously, Dings et al. reported that OTX008 inhibits endothelial cell proliferation and migration *in vitro* and reduces tumour growth and angiogenesis in multiple xenograft models [39,40].

The goal of this study was to elucidate the mechanism of action of OTX008 by evaluating its direct antitumour effects using human cancer cells and xenograft models. We examined the effect of OTX008 on expression of Gal1 and various downstream molecules. We also analysed the role of Gal1 inhibition in cell cycle arrest and cell invasion *in vitro*. Effects of Gal1 inhibition on *in vitro* proliferation was confirmed by *in vivo* experiments. Finally, to optimally position OTX008 in clinical development, OTX008 combination with conventional chemotherapy and targeted therapies agents was evaluated for potential synergy and optimal sequence administration.

2. Materials and methods

2.1. Principal reagents and materials

See Supplementary Materials and methods for detail.

2.3. Cell lines

SKBR3, MCF7, SKOV3, CAKI1, HT29, OVCAR3, DU145, IGROV1, SK-HEP1, ZR-75-1, PC3 and normal fibroblasts were obtained from the ATCC (Rockville, United States of America). HCT116, COLO205-S, HCC2998, HOP62 and HOP92 cell lines were obtained from the National Cancer Institute collection. SCC61, HEP2 and SQ20B were provided by Eric Deutsch (Gustave Roussy Institute, Villejuif, France).

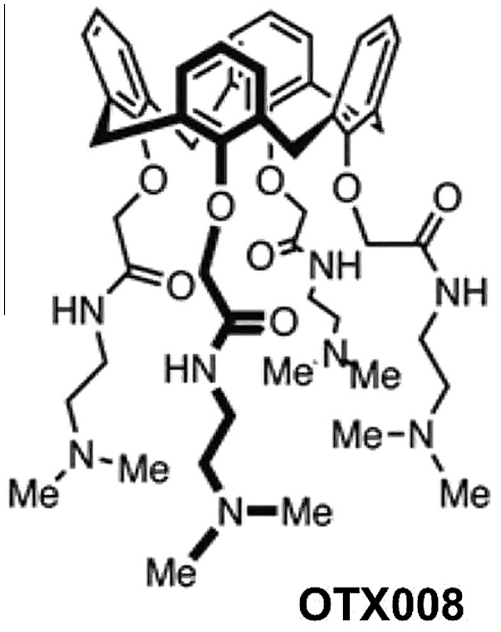

Fig. 1. Chemical structure of OTX008.

A2780-1A9 was provided by Maurizio D'Incalci (Mario Negri Institute, Milan, Italy). Mesenchymal COLO205-R (adenocarcinoma colon cell line) was developed in our laboratory from the parental epithelial COLO205-S cell line [40]. A mesenchymal MCF7-sh*WISP* breast cancer cell was obtained by transfection of a WISP-targeted shRNA in MCF7 breast cancer cells [41] (provided by Michel Sabbah, Saint-Antoine Hospital, Paris, France). Cells were grown in RPMI medium supplemented with 10% foetal calf serum (FCS, Invitrogen, Cergy-Pontoise, France), 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere, and checked regularly for the absence of *Mycoplasma*.

2.4. In vitro cell proliferation assay

See Supplementary Materials and methods for detail.

2.5. Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

See Supplementary Materials and methods for detail.

2.6. Cell cycle analysis

See Supplementary Materials and methods for detail.

2.7. Western blot analysis

See Supplementary Materials and methods for detail.

2.8. Small hairpin RNA (shRNA)

See Supplementary Materials and methods for detail.

2.9. Matrigel invasion assay

See Supplementary Materials and methods for detail.

2.10. Mouse ovarian xenograft model

A total of 8×10^6 A2780-1A9 ovarian cells were injected subcutaneously into the right lateral flank of female *nu/nu* athymic mice (Harlan Laboratories, Indiana, United States of America). Once tumours were palpable (50 mm^3), mice were randomised to receive treatment intraperitoneally with either PBS (3 times/week), 5 mg/kg OTX008 (3 times/week), 6 mg/kg cisplatin (days 1, 8 and 15) or 10 mg/kg docetaxel (days 1, 8 and 15). Tumour size was measured twice weekly with calipers and tumour volume was calculated as $3.14 \times (\text{width}^2)/\text{length}$. Mice were sacrificed after 2 weeks and tumours stored in Tissue-Tek® OCT (Sakura finetek, Flemingweg, Netherlands). Animal experiments were approved by the Animal Housing

and Experiment Board of the French Health Authorities.

2.11. Immunohistochemistry

See Supplementary Materials and methods for detail.

2.12. Combination studies

For combination studies, we used three different administration schedules for each drug and each cell line; concomitant (two drugs together), sequential 1 (drug A, washout, drug B) and sequential 2 (drug B, washout, drug A).

For concomitant exposure, cells were seeded and treated 24 h later with increasing OTX008 concentrations alone or combined with different concentrations of a second drug. For sequential exposure, cells were seeded and allowed to grow for 24 h, exposed to various concentrations of the first drug, washed and the second drug was added. Concentrations were determined according to the GI50 values for a single dose. Based on GI50 for each drug as single agent summarised in Supplementary Table 1, drug concentrations employed for combinations studies correspond to GI_{20}, GI_{40}, GI_{60} and GI_{80}. The growth inhibitory effect was determined using MTT assay. To assess drug–drug interaction, dose–response data were evaluated with the CalcuSyn program (Biosoft, Cambridge, United Kingdom) which applies median effect methodology developed by Chou and Talalay [42]. This algorithm estimates a combination index (CI) for each data point based on the results expected from each single agent. If the experimental effects of combination are greater than expected, the CI value will be less than 1, reflecting synergy. Whereas, if the experimental effects of combination are less than expected, the CI value will be greater than 1, indicating antagonism. CI values between 1 and 1.1 are considered to reflect additive effects. Statistical analysis and graphs were carried out using Instat and Prism software (GraphPad, San Diego, CA, United States of America).

2.13. Statistical analysis

Results are expressed as mean ± standard deviation of at least three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) a posteriori test or by two-way ANOVA followed by Bonferroni a posteriori test or employing Prism 5.00 for MS Windows software (Graph Pad Software, San Diego, CA, United States of America). A p-value of 0.05 or less was considered statistically significant. The strength of the linear association between two variables was quantified using the correlation coefficient R; R^2 represents the square of the correlation coefficient in linear regression.

3. Results

3.1. Correlation of OTX008 antiproliferative effects with Gal1 expression in cancer cell lines

OTX008 antiproliferative effects were assessed *in vitro* in a large panel of human solid tumour cell lines which were characterised for RNA expression of relevant molecules. Growth inhibitory concentrations (GI_{50}) ranged from 3 to 500 μM (Table 1). Higher concentrations of anginex were required to obtain equivalent OTX008 anti-proliferative effects (>300 μM GI_{50} in most cell lines evaluated).

We observed a significant correlation between OTX008 GI_{50} values and Gal1 mRNA (*LGALS1*) and protein expression levels in our panel of cancer cells (Fig. 2A). However, no correlations were observed between OTX008 sensitivity and galectin-3 (*LGALS3*), galectin-8 (*LGALS8*), semaphorin-3A (Sema3A), semaphorin-3B, neuropilin-1 (NRP-1), vascular endothelial growth factor receptor 1–3 (VEGFR) and vascular endothelial growth factor A–D (VEGF) mRNA levels in the panel of cell lines (Table 1). Moreover, OTX008 did not affect the proliferation of normal cultured fibroblasts which have high *LGALS1* levels (Table 1).

3.2. Epithelial-to-mesenchymal phenotype and OTX008 sensitivity

As shown in Fig. 2B, we observed that higher *LGALS1* levels were strongly correlated with several mesenchymal markers such as vimentin (*VIM*) and N-cadherin (*CDH2*) and low mRNA levels of epithelial markers as E-cadherin (*CDH1*) in our panel of cell lines. We evaluated OTX008 antiproliferative effects in two paired epithelial-mesenchymal models, COLO205-S/COLO205-R and MCF7/MCF7-sh*WISP* (Fig. 2C). COLO205-S cells were more sensitive to OTX008 than COLO205-R cells ($GI_{50} = 52$ versus > 300 μM, respectively) and similarly, OTX008 demonstrated more potent antiproliferative effects in the epithelial MCF7 model than its mesenchymal counterpart MCF7-sh*WISP* after 72 h-exposure.

3.3. Effect of OTX008 on cell migration, cell cycle progression and survival signalling cascades

The pharmacodynamics of OTX008 were evaluated in two cell lines, SQ20B (H&N) and A2780-1A9 (ovarian) with high and intermediate sensitivity to OTX008, respectively (Table 1).

Analysis of the cell cycle in SQ20B and A2780-1A9 cells revealed that OTX008 exposure increased the

Table 1
72 h-antiproliferative effects of OTX008 and Anginex in a panel of human cancer cell lines characterised for mRNA expression of selected genes. Results are expressed as '*n*-fold differences' in target gene expression relative to housekeeping TBP gene. ND, not determined. H&N, Head and Neck; NSCL, non-small cell lung carcinoma; HCC, hepatocellular carcinoma.

Cell line	Tumour type	GI50 (μM) OTX008		GI50 (μM) Anginex	mRNA relative expression												
		Mean	SD		*LGALS1*	*LGALS3*	*LGALS8*	*SEMA3A*	*SEMA3B*	*NRP1*	*VEGFR1*	*VEGFR2*	*VEGFR3*	*VEGFA*	*VEGFB*	*VEGFC*	*VEGFD*
SQ20B	H&N	3	1.2	>300	47,434	110,801	27,053.6	8995	11,074	4073	203	19	53	1294	4152	4158	21
HT29	Colon	50	0.7	>300	3639	17,054	8871.93	509	5063	867	12	0	8	400	5359	0	14
COLO205-S	Colon	52	5.1	>300	108,817	68,250	22,673.3	2	23	1068	9	0	13	186	3458	0	13
HCC2998	Colon	63	4.9	ND	6213	231,447	5655.5	51	9100	502	4	0	10	883	2629	0	4
SCC 61	H&N	82	15.2	ND	135,675	12,586	8555.57	1470	3580	821	6	0	30	1438	5145	1522	7
HOP92	NSLC	95	5.9	ND	424,492	30,049	25,726	137	3869	31,051	17	5	18	1117	7296	5596	39
MCF7	Breast	98	10.3	>300	251,341	38,101	9045.48	1588	1875	752	0	78	5	737	1918	12	9
SK BR3	Breast	100	3.2	>300	66,860	69,847	28,284.9	0	6985	1938	94	0	28	416	21,514	345	18
HCT116	Colon	130	14.3	>300	149,274	21,225	5824.23	255	931	645	4	5	4	182	2649	0	47
A2780-1A9	Ovarian	170	12.3	ND	9244	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ZR-75-1	Breast	185	9.2	ND	706	41,561	50,612.9	0	186	115	3	2	6	327	1951	884	43
PC3	Prostate	210	23.6	>300	206,652	36,117	16,727.1	2224	4615	195,937	2	29	18	1231	4034	5349	19
CAKI 1	Renal	218	7.4	>300	359,275	59,746	7190.69	752	149	6073	12	6	11	431	7219	4222	56
OVCAR 3	Ovarian	230	65.0	>300	362,858	32,039	11,761.3	6095	1885	451	13	24	10	522	1716	0	16
SK-HEP1	HCC	232	12.0	>300	504,590	10,831	11,624.6	1293	56	10,463	1	0	5	180	4358	2498	1
HEP2	H&N	260	18.2	>300	210,303	35,834	12,070.6	22	15,559	694	4	0	15	1946	7032	450	5
HOP62	NSLC	>300		ND	511,711	58,097	16,009.4	1784	4839	16,525	104	9	9	1850	10,417	7044	36
COLO205-R	Colon	>300		>300	370,962	17,400	9841.99	2813	4221	240	5	24	7	995	2771	0	33
DU 145	Prostate	>300		280	290,914	25,332	6911.67	21	87	13,463	5	0	167	2099	0	1337	24
IGROV1	Ovarian	>300		ND	685,395	5577	4776.42	3	3103	1729	5	0	11	704	5448	0	253
MCF7-shWISP	Breast	>300		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SKOV3	Ovarian	>300		ND	692,784	98,884	10,323	4870	6519	27,762	17	244	34	1977	15,095	1468	205
Normal fibroblasts		>300		ND	980,185	50,597	6683	15	597	12,556	1092	767	228	2639	9561	7746	8

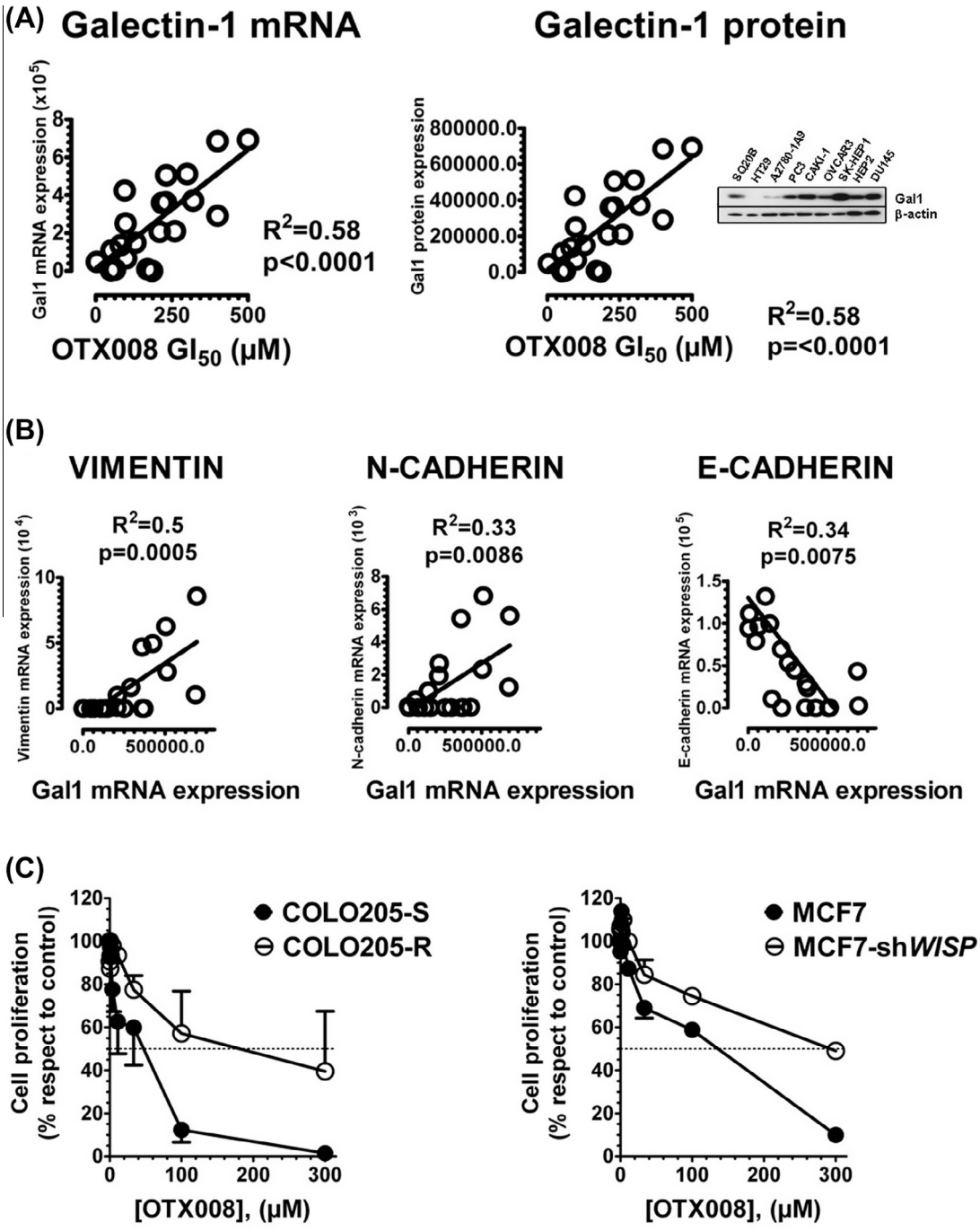

Fig. 2. OTX008 antiproliferative effects correlate with Galectin-1 (Gal1) expression and epithelial-to-mesenchymal transition (EMT) phenotype of a wide range of human cancer cell lines. (A) Correlation between OTX008 GI_{50} and *LGALS1* mRNA levels or Gal1 protein expression. Insert: Western Blot of Gal1 in cancer cell lines displaying different sensitivity to OTX008. (B) Correlation between *LGALS1* and vimentin, E-cadherin (*CDH1*) and N-cadherin (*CDH2*) relative mRNA expression. (C) Antiproliferative effects of OTX008 in two paired EMT models, COLO205-S/COLO205-R and MCF7/MCF7-sh*WISP* after 72 h-treatment.

proportion of cells in the G2/M phase while the proportion in the subG1 phase was not altered after 72 h-exposure (Fig. 3A and B). The mechanism of OTX008-induced G2/M arrest was investigated in terms of various proteins which control cell cycle arrest at G2/M by modulating CDK1 activity. In SQ20B cells, 72-h exposure to 3 µM OTX008 resulted in increased phosphorylation of the inhibitory residue Tyr^{15} of CDK1, together with decreased p-$CDC25C^{ser216}$, a CDK1 phosphatase, and p-$WEE1^{ser642}$ a CDK1 inhibitor (Fig. 3C). Following OTX008 exposure, we observed a transient upregulation of p-$ERK1/2^{thr202/tyr204}$ and p-AKT^{ser473} protein levels

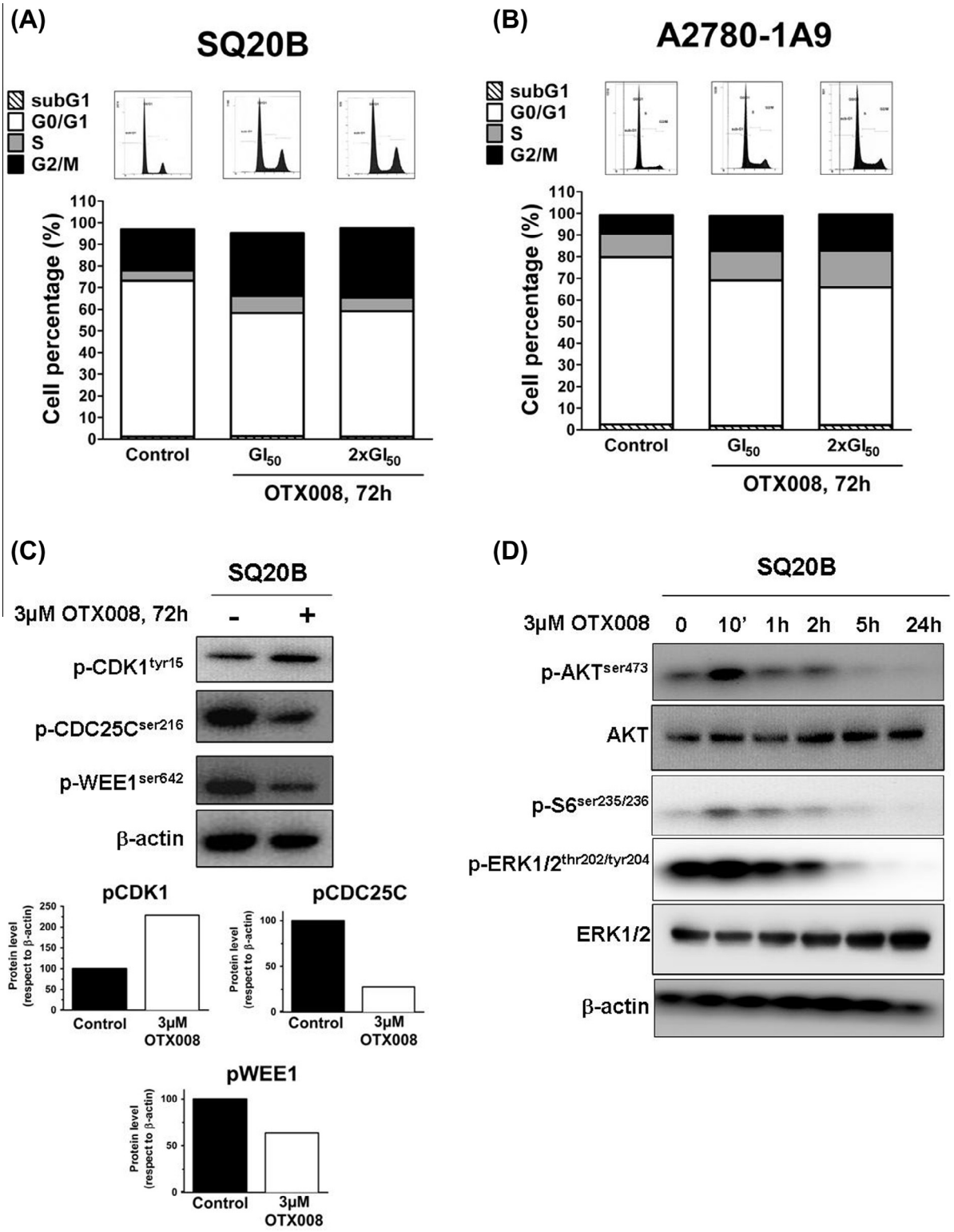

Fig. 3. OTX008 effects on cell cycle and survival signaling pathways. Effects of 72 h exposure to GI_{50} and $2\times GI_{50}$ OTX008 on the cell cycle of SQ20B (A) and A2780-1A9 (B) cells. Results are representative of two independent experiments. (C) Protein modulation of p-CDK1^{tyr15}, p-CDC25C^{ser216} and p-WEE1^{ser642} in SQ20B cells treated with 3 μM OTX008 for 72 h. (D) Modulation of p-AKT^{ser473}, AKT, p-S6$^{ser235/236}$, p-ERK1/2$^{thr202/tyr204}$ and ERK1/2 in SQ20B cells treated with 3 μM OTX008 at different time points.

followed by a sustained inhibition in SQ20B after 2 h-treatment (Fig. 3D) and A2780-1A9 cells (data not shown).

These findings indicate that OTX008 antiproliferative effects are associated with cell-cycle and survival pathways modulation.

3.5. Effect of OTX008 on Gal1 protein levels

Exposure to 3 μM OTX008 decreased Gal1 protein expression in a time-dependent manner in SQ20B cells ($p < 0.01$ at 48 h relative to baseline; Fig. 4A), despite no significant changes in *LGALS1* mRNA levels (data not shown). Similar results were observed in A2780-1A9 ovarian cells after 24 h, 48 h and 72 h exposure to 170 μM OTX008 (Fig. 4B). However, exposure to 3 μM OTX008 up to 72 h did not modulate galectin-3 protein levels in SQ20B cells (Fig. 4C), reflecting the specificity of OTX008 for Gal1.

3.6. Effect of OTX008 treatment or Gal1 silencing on cell invasion

Stable transfection of SQ20B cells with a small hairpin *LGALS1*-RNA (SQ-shGAL1) was performed to evaluate whether Gal1 silencing reproduces OTX008 effects. SQ-shGAL1 cells displayed a significant decrease in Gal1 protein expression without difference in cell doubling time compared to control scrambled shRNA transfected cells (Fig. 4D). As shown in Fig. 4E, 3 μM OTX008 treatment induced a significant inhibition of *in vitro* invasion of SQ20B cells after 48 h ($p < 0.01$ relative to untreated cells), and sh*LGALS1*-RNA transfected cells had lower invasion rates than control cells.

3.7. Effect of OTX008 on Gal1 extracellular binding partners

To identify if OTX008 affects the carbohydrate-binding activity of Gal1 to extracellular matrix components, SQ20B cells were grown on fibronectin- and laminin-coated or uncoated plates and GI_{50} values were determined after 72-h exposure. Outcomes were similar for all three experimental conditions pointing out that OTX008 antiproliferative effects are not related to the interaction of Gal1 with components of the extracellular matrix (Fig. 5A). Similar results were observed for HT29 cells (data not shown), an adenocarcinoma colon model with intermediate OTX008 sensitivity (Table 1).

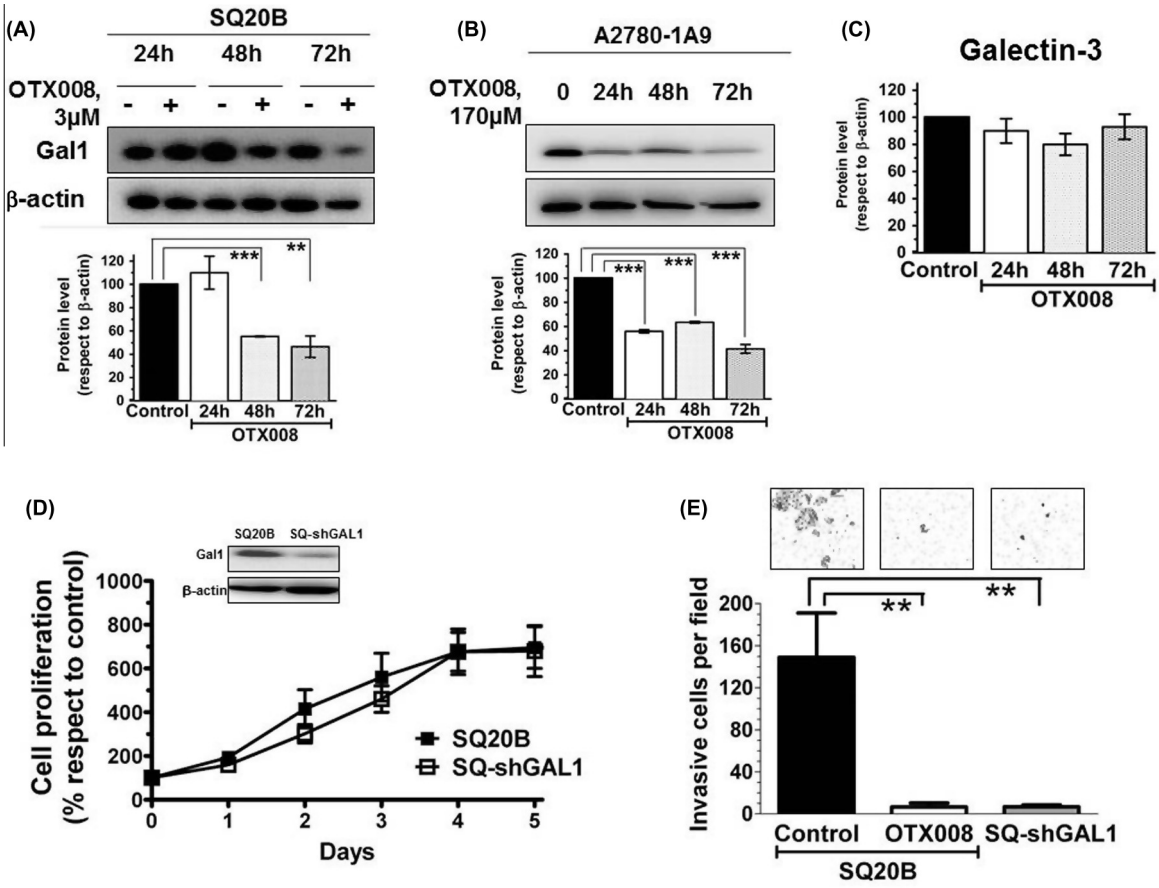

Fig. 4. Effects of OTX008 on Galectin-1 (Gal1) protein expression and SQ20B-Matrigel invasion.(A) Gal1 levels in SQ20B cells treated for 24, 48 and 72 h with 3 μM OTX008. $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Gal1 levels in A2780-1A9 cells treated for 24, 48 and 72 h with 170 μM OTX008, respectively; $^{***}p < 0.001$. (C) Galectin-3 expression in SQ20B cells treated with 3 μM OTX008 for 24, 48 and 72 h. (D) *In vitro* proliferation assay on SQ20B transfected with Gal1 shRNA (SQ-shGAL1) or scrambled shRNA (SQ20B). Insert: Effect on Gal1 levels in SQ20B cells transfected with Gal1 shRNA (SQ-shGAL1) or scrambled shRNA. (E) *In vitro* Matrigel invasion assay of SQ20B cells treated or not with 3 μM OTX008 and of SQ-shGAL1 cells, quantified after 48 h by counting the average number of invading cells. $^{**}p < 0.01$.

Detectable levels of mRNA (Table 1) and protein levels (data not shown) of NRP-1 and Sema3A were observed in SQ20B cells. Recombinant Sema3A (10 ng/mL) and recombinant Gal1 (100 ng/mL) induced transient activations in p-AKT^{ser473} and p-ERK1/2, suggesting that the Gal1/Sema3A system is functional in this cell line (Fig. 5B, insert). SQ20B cells were thus selected to evaluate the effects of OTX008 on the Gal1/Sema3A/NRP-1 pathway in cancer cells.

After 3 day exposure, recombinant Gal1 had no effect on SQ20B proliferation, whereas exogenous Sema3A inhibited SQ20B proliferation in a dose-dependent manner (Fig. 5B). OTX008 slightly synergised the antiproliferative effects of Sema3A, suggesting that it affects Gal1/NRP-1 binding (Fig. 5B). As shown in Fig. 5C, recombinant Gal1 enhanced invasion of SQ20B cells after 48-h exposure, which was counteracted by 3 μM OTX008 at 48 h. Notably, Sema3A also increased SQ20B-invasion, again inhibited by OTX008. Our findings suggest that OTX008 inhibited SQ20B-cell invasion induced by recombinant Gal1 or enhanced Sema3A antiproliferative effects probably through inhibition of the Gal1/NRP-1 interaction in cancer cells.

3.8. Effect of OTX008 on cancer cell proliferation and angiogenesis in tumour-bearing mice

OTX008 *in vivo* efficacy was evaluated in nude mice bearing A2780-1A9 ovarian cancer xenografts, along with cisplatin and docetaxel. OTX008 doses yielding plasma concentrations in the range of GI_{50} concentrations used in cultured cells were chosen (data not shown). Tumour growth was significantly inhibited after 2 weeks of OTX008 treatment ($p < 0.05$), and to a similar extent as with cisplatin and docetaxel (Fig. 6A). However, in contrast to cisplatin and docetaxel, no significant weight loss or overt signs of toxicity were observed with OTX008 treatment (data not shown).

We also showed that tumour growth inhibition *in vivo* was associated with a significant reduction of nuclear Ki67 staining in OTX008-treated tumours compared to vehicle-treated tumours (Fig. 6B). A significant reduction of the vascular area and decreased expression of VEGFR-2 was observed after OTX008 *in vivo* treatment compared to vehicle (Fig. 6B), suggesting anti-angiogenic effects of Gal1 inhibition on A2780-1A9 xenografts.

3.9. Antiproliferative synergy of OTX008 with chemotherapeutic and targeted therapies

Combination studies with OTX008 were performed using conventional cytotoxic drugs and receptor tyrosine kinases inhibitors (RTKi) with a broad range of mechanisms of actions, including gefitinib, sunitinib, sorafenib and regorafenib, and everolimus (an m-TOR inhibitor). Colon, head and neck (H&N) and hepatocellular carcinoma (HCC) cell lines were evaluated using 48 h or 72 h- sequential and concomitant combination schedules, where combination Index (CI) was determined using Chou and Talalay analysis. OTX008 combined with cisplatin exerted synergistic effects in wild-type K-RAS colon cancer cells (COLO205-S and HT29) and antagonistic/additive effects in mutated K-RAS colon cancer cells (COLO205-R and HCT116; Table 2), whereas additive effects were observed in three of four H&N cell lines with the three different administration schedules (Table 3). Interestingly, OTX008 combined with oxaliplatin was synergistic in all colon (Table 2) and H&N (Table 3) cell lines tested regardless of the 48 h-exposure schedule used, while it was additive in HCC cell lines (Table 4). Combined OTX008 and carboplatin were mainly antagonistic in H&N cells (Table 3). Synergy was observed after sequential OTX008 exposure with 5-FU and 5′-DFUR and OTX008 exposure prior to gemcitabine in colon cancer cell lines (Table 2). Combined OTX008 and gemcitabine was additive in HCC cells (Table 4). Interestingly, all administration schedules with combined OTX008 and docetaxel were synergistic in colon and H&N cell lines (Tables 2 and 3).

In H&N cell lines, all administration schedules combining OTX008 and gefitinib were additive after 48 h (Table 3). OTX008 24 h-exposure prior to sunitinib displayed synergistic effects while the reverse sequence was additive in all colon, H&N and HCC cancer cell lines (Tables 2–4). Unlike sunitinib, sorafenib and OTX008 exhibited mostly antagonistic or additive effects in colon and HCC cell lines (Tables 2 and 4). In addition, OTX008 treatment before regorafenib was synergistic in all colon cancer cell lines, while the other exposure administration schedules had antagonistic or additive effects (Table 2). In addition, OTX008 in combination simultaneously with an m-TOR inhibitor had synergistic antiproliferative effects in HCC cancer cell lines after 72 h-exposure (Table 4).

In summary, our results indicate that OTX008 enhanced the *in vitro* antiproliferative effects of several cytotoxic drugs including cisplatin, oxaliplatin, docetaxel, 5-FU and 5′-DFUR, targeted therapies including sunitinib and everolimus, or the novel approved RTKi regorafenib, primarily when OTX008 was administered first, indicating that combinations maybe considered an important aspect of OTX008 clinical development.

4. Discussion

This study contributes to our current understanding of the role of Gal1 in cancer development, providing mechanistic insights into direct roles it can play, and also helps elucidate the mechanism of action of the novel small molecule Gal1-inhibitor, OTX008. We show here

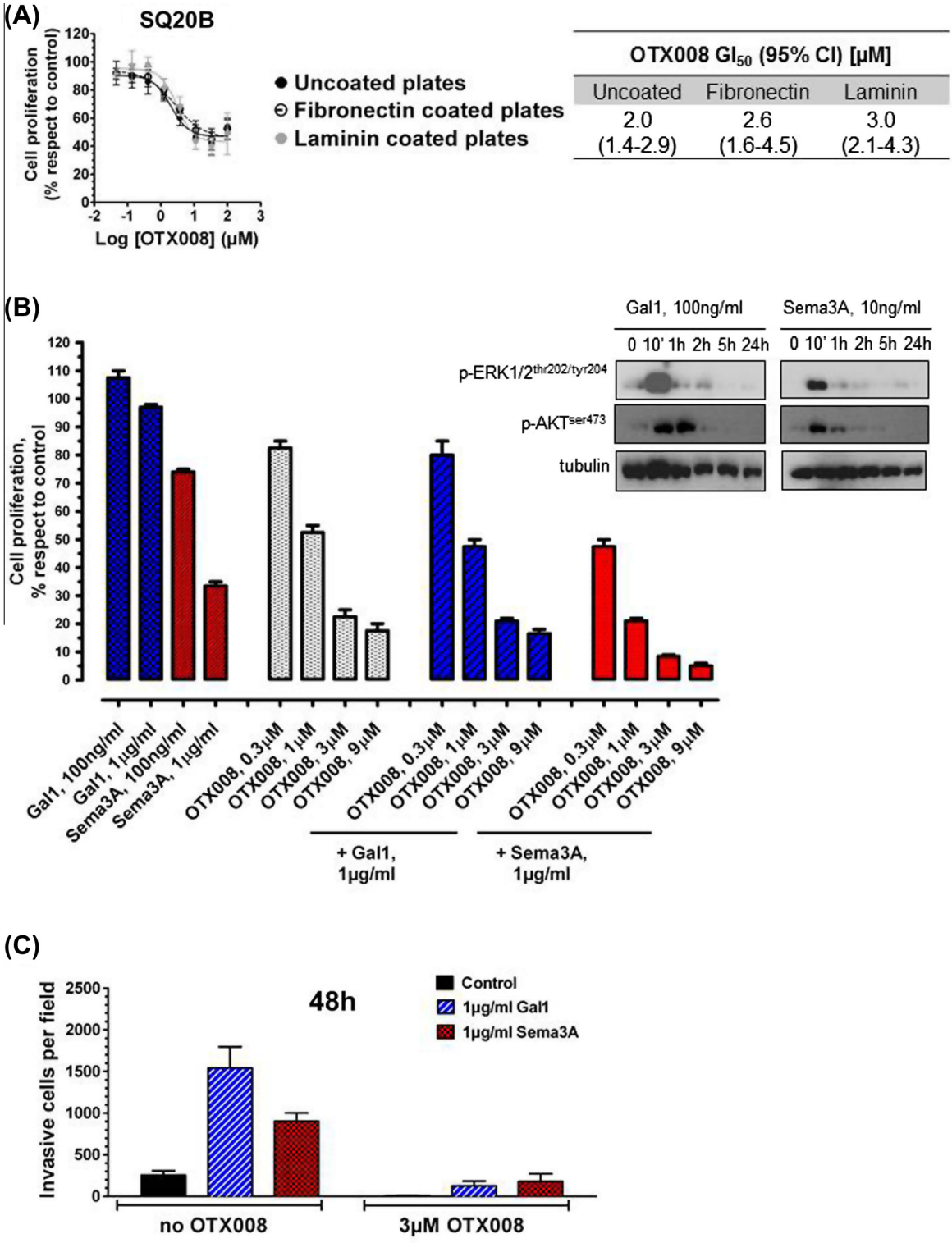

Fig. 5. Effects of OTX008 on Galectin-1 (Gal1) extracellular binding partners. (A) Antiproliferative effects of 72 h OTX008 exposure in SQ20B using uncoated and laminin or fibronectin-coated plates. (B) Effects of Gal1, Semaphorin-3A (Sema3A), and lactose, with or without different OTX008 concentrations on SQ20B growth after 3 days of treatment. Insert: Modulation of p-ERK1/2$^{thr202/tyr204}$ and p-AKT^{ser473} on SQ20B cells after exposure to 100 ng/mL Gal1 or 10 ng/mL Sema3A. (C) *In vitro* Matrigel invasion assay of SQ20B exposed to 1 μg/mL Gal1, 1 μg/mL Sema3A or 200 ng/mL lactose with or without 3 μM OTX008. Invasion was quantified after 48 h by counting the average number of invaded cells.

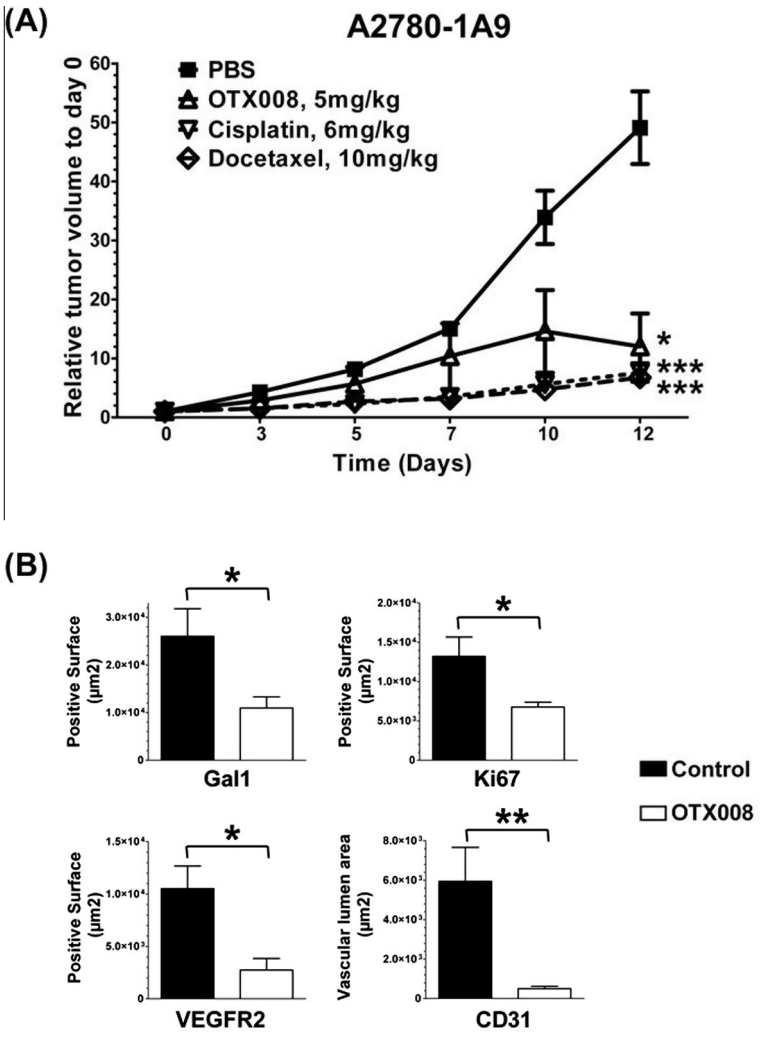

Fig. 6. Antitumour and antiangiogenic effects of OTX008 in A2780-1A9 human tumour xenografts. (A) Antitumour effects (tumour volume) of intraperitoneal 5 mg/kg OTX008 on A2780-1A9 xenograft were compared to those of active doses of 6 mg/kg cisplatin and 10mg/kg docetaxel. $^{*}p < 0.05$, $^{***}p < 0.001$. (B) Immunohistochemistry of Galectin-1 (Gal1), Ki67, VEGFR2 and CD31 on A2780-1A9 tumours after 2 weeks of OTX008 treatment compared to vehicle-treated animals. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 2
OTX008 *in vitro* combination studies in colon cancer cell lines. Median combination index (95% confidence interval) for (A) OTX008 + drug for 48 h, (B) OTX008 24 h then drug 24 h, (C) Drug 24 h then OTX008 24 h. Results from 3 independent experiments. Combination index (CI) < 1: synergy (orange); CI = 1–1.1: additivity (blue); CI > 1.1: antagonism (white). ND, not determined.

Cell lines	Schedules	Median combination index (95% confidence interval)									
		Cisplatin	Oxaliplatin	5-FU	5'-DFUR	Gemcitabine	Docetaxel	Sunitinib	Sorafenib	Regorafenib	
COLO205-S	A	0.6 (0.3-1.2)	0.6 (0.3-1.2)	1.6 (0.4-2.5)	1.5 (0.6-2.0)	1.5 (0.4-2.6)	1.0 (0.7-1.2)	1 (0.6-1.7)	1.2 (0.7-3.8)	1.3 (0.7-4.4)	KRAS WT
	B	0.4 (0.2-1.6)	0.5 (0.3-1.7)	0.8 (0.2-2.0)	0.6 (0.3-1.4)	1.5 (0.5-2.5)	0.7 (0.6-0.9)	0.8 (0.3-1.4)	1.1 (0.8-2.8)	0.8 (0.5-0.8)	
	C	0.6 (0.3-1.4)	0.7 (0.1-1.7)	0.9 (0.3-2.3)	0.8 (0.4-1.3)	0.7 (0.5-1.4)	0.6 (0.6-1.7)	1 (0.2-1.6)	0.9 (0.5-1.3)	1.1 (0.5-1.9)	
HT29	A	0.8 (0.3-1.4)	0.5 (0.2-0.8)	1.0 (0.4-1.6)	1.0 (0.7-1.9)	0.8 (0.4-1.7)	0.8 (0.5-1.4)	1 .1(0.3-2.2)	1.0 (0.6-1.5)	1.0 (0.3-1.6)	
	B	0.5 (0.1-1.3)	0.2 (0.1-0.7)	0.4 (0.1-1.2)	0.5 (0.2-1.5)	0.1 (0.01-1.5)	0.8 (0.5-1.5)	0.8 (0.3-1.3)	1.2 (0.6-1.6)	0.8 (0.3-1.4)	
	C	0.6 (0.2-0.9)	0.7 (0.3-1.1)	0.8 (0.2-1.6)	0.5 (0.2-1.0)	0.6 (0.4-1.2)	0.9 (0.5-1.1)	0.9 (0.3-1.2)	0.8 (0.5-1.4)	0.8 (0.4-1.3)	
COLO205-R	A	0.9 (0.4-4.8)	0.3 (0.1-1.7)	0.9 (0.6-2.9)	0.8 (0.4-4.3)	1.2 (0.5-3.7)	ND	0.9 (0.5-4.8)	0.9 (0.6-2.0)	1.1 (0.6-1.6)	KRAS MUTATED (exon 2, G13D hétérozygous)
	B	0.8 (0.4-1.7)	0.3 (0.1-0.6)	0.9 (0.4-4.8)	0.4 (0.2-2.0)	0.8 (0.4-1.5)	ND	0.5 (0.3-0.8)	1.2 (0.5-1.9)	0.7 (0.5-2.7)	
	C	1.1 (0.4-4.1)	0.4 (0.3-1.3)	0.4 (0.2-1.2)	0.7 (0.3-4.2)	1.5 (0.6-4.0)	ND	0.8 (0.6-2.2)	1.0 (0.6-3.7)	0.8 (0.5-1.8)	
HCT116	A	1.5 (0.5-3.0)	0.6 (0.1-1.5)	1.1 (0.5-1.9)	0.8 (0.3-3.0)	1.1 (0.4-2.1)	ND	0.9 (0.6-2.6)	1.3 (0.7-5.2)	2.4 (0.4-4.1)	
	B	1.1 (0.4-1.9)	0.4 (0.1-0.7)	0.6 (0.3-1.1)	1 (0.6-1.3)	0.9 (0.4-1.8)	ND	0.6 (0.4-0.9)	1.4 (0.4-3.9)	0.6 (0.3-2.7)	
	C	1.7 (0.7-6.3)	0.8 (0.2-2.9)	0.2 (0.2-1.8)	0.6 (0.3-1.8)	1.7 (0.8-6.3)	ND	0.7 (0.5-1.0)	1.1 (0.7-3.9)	1.3 (0.4-1.8)	

Table 3
OTX008 *in vitro* combination studies in head and neck cancer cell lines. Median combination index (95% confidence interval) for (A) OTX008 + drug for 48 h, (B) OTX008 24 h then drug 24 h, (C) drug 24 h then OTX008 24 h. Results from 3 independent experiments. CI < 1: synergy (orange); Combination index (CI) = 1–1.1: additivity (blue); CI > 1.1: antagonism (white). ND, not determined.

Cell lines	Schedules	Median combination index (95% confidence interval)					
		Cisplatin	Oxaliplatin	Carboplatin	Docetaxel	Gefitinib	Sunitinib
SQ20B	A	1.0 (0.1-2.6)	0.5 (0.1-4.2)	1.4 (0.7-2.9)	0.9 (0.1-3.8)	1.1 (0.3-2.7)	1.0 (0.6-5.8)
	B	1.0 (0.3-3.2)	0.3 (0.1-1.6)	1.5 (0.2-2.7)	0.7 (0.1-4.6)	0.9 (0.2-2.3)	0.5(0.3-0.9)
	C	1.0 (0.6-3.6)	0.6 (0.1-2.1)	1.2 (0.5-2.1)	0.5 (0.01-7.8)	1.0 (0.3-1.4)	1.0 (0.8-1.9)
HEP2	A	1.3 (0.4-3.8)	1.1 (0.5-2.3)	1.5 (0.7-2.8)	1.6 (0.4-5.3)	1.0 (0.2-2.3)	0.8 (0.4-1.9)
	B	1.1 (0.6-2.2)	0.6 (0.4-1.5)	1.2 (0.4-2.4)	1.1 (0.03-3.8)	0.9 (0.3-1.8)	0.8 (0.1-2.6)
	C	0.8 (0.5-1.8)	0.8 (0.1-2.5)	1.1 (0.7-1.8)	1.4 (0.3-22.2)	1.1 (0.4-2.3)	0.4 (0.2-2.0)
SCC61	A	1.0 (0.3-2.6)	ND	1.1 (0.2-2)	0.8 (0.3-3.2)	0.8 (0.2-2.6)	ND
	B	1.0 (0.1-3.4)	ND	1.2 (0.2-1.9)	0.9 (0.3-1.9)	1.0 (0.3-2.2)	ND
	C	1.0 (0.5-1.5)	ND	1.0 (0.4-2.5)	0.5 (0.05-3.2)	1.1 (0.6-2.1)	ND
DETROIT	A	0.9 (0.3-1.8)	ND	ND	0.6 (0.1-4.3)	1.2 (0.7-1.8)	ND
	B	0.9 (0.4-2.0)	ND	ND	0.5 (0.3-2.4)	0.8 (0.4-2.1)	ND
	C	1.2 (0.3-3)	ND	ND	0.8 (0.2-8.5)	0.8 (0.6-1.3)	ND

that OTX008 exhibits direct antitumour activity *in vitro* by inhibiting cell cycle progression, cell invasion, and proliferation, and also by enhancing the antiproliferative effects of several cytotoxic and targeted therapy agents. These findings were supported by *in vivo* studies showing that OTX008 delayed tumour growth in human ovarian cancer xenografts.

Expression of Gal1 mRNA and protein directly correlate with OTX008 concentrations required to inhibit cellular proliferation in cancer cells, which is consistent with reports that OTX008 binds allosterically to Gal1 in a dose-dependent manner [38]. OTX008 concentrations needed to inhibit proliferation were proportional to Gal1 mRNA and protein levels in a range of cancer cell lines. Several studies have shown that Gal1 plays an important role in invasion and migration [8,25], both of which are increased in cells having undergone EMT. Coherent with this, we found higher Gal1 expression in cancer cells with a mesenchymal phenotype compared to those which were epithelial. In turn, OTX008 displayed antiproliferative effects at low concentrations in cancer cells with an epithelial phenotype, but required higher concentrations to inhibit the growth of cancer cells displaying a mesenchymal phenotype. This highlights the importance of a cancer cell's epithelial status in terms of OTX008 sensitivity.

OTX008 directly affected cancer cell proliferation in a dose-dependent manner via mechanisms modulating cell cycle progression and survival signalling pathways. G2/M cell cycle arrest was induced by modulating activity of CDK1 via CDC25C and WEE1, proteins regulating the G2/M checkpoint. Previous reports suggest that intracellular Gal1 might favour the H-Ras- and K-Ras-GTP conformations, which modulate signal

Table 4
OTX008 *in vitro* combination studies in hepatocarcinoma cell lines. Median combination index (95% confidence interval) for (A) OTX008 + drug, (B) OTX008 then drug, (C) drug then OTX008, according to (1) Simultaneous exposure for 72 h and sequential exposure for 36 h then 36 h or (2) Simultaneous exposure for 48 h and sequential exposure for 24 h then 24 h. Results from 3 independent experiments. Combination index (CI) < 1: synergy (orange); CI = 1–1.1: additivity (blue); CI > 1.1: antagonism (white).

Cell lines	Schedules	Median combination index (95% confidence interval)				
		Oxaliplatin (2)	Gemcitabine (2)	Sunitinib (1)	Sorafenib (1)	Everolimus (1)
SK-HEP1	A	1.2 (0.6-2.8)	0.8 (0.1-6.9)	1.4 (0.5-2.8)	0.8 (0.3-2.7)	0.8 (0.2-2.3)
	B	0.8 (0.1-2)	1.0 (0.2-2.8)	1.0 (0.6-2.0)	1.4 (0.7-6.7)	1.3 (0.3-3.8)
	C	1 (0.5-2.4)	0.7 (0.1-6.4)	1.6 (0.6-8.7)	0.5 (0.1-1.8)	1.1 (0.3-2.3)
HEP3B	A	1.4 (0.2-3.4)	1.3 (0.2-8.9)	1.2 (0.5-3.0)	1.2 (0.6-2.2)	0.8 (0.1-1.5)
	B	1.2 (0.3-4.7)	0.9 (0.2-2.9)	0.7 (0.16-1.6)	0.7 (0.3-1.7)	0.9 (0.2-1.8)
	C	1.1 (0.4-2.8)	1.2 (0.2-26.8)	1.2 (0.6-4.8)	1.2 (0.5-2.1)	1.1 (0.3-2.5)
HEPG2	A	1 (0.3-2.3)	1.1 (0.5-2.3)	1.3 (0.5-3.0)	0.9 (0.5-1.8)	0.9 (0.5-1.4)
	B	0.9 (0.1-9.2)	0.9 (0.2-4.3)	0.6 0.3-2.2)	0.7 (0.1-1.8)	1.2 (0.5-2.4)
	C	0.9 (0.3-3.2)	0.6 (0.1-3.6)	1.0 (0.3-1.6)	0.6 (0.3-2.5)	1.0 (0.4-1.5)
JHH6	A	1.4 (0.2-3.4)	1.2 (0.2-3.7)	0.9 (0.6-2.6)	1.0 (0.5-2.1)	0.6 (0.5-1.5)
	B	0.9 (0.3-2.3)	0.9 (0.3-2.3)	0.5 (0.5-1.6)	1.1 (0.3-2.7)	0.8 (0.4-2.4)
	C	1.3 (0.6-2.8)	1 (0.4-1.7)	0.9 (0.5-3.9)	1.3 (0.6-4.2)	1.3 (0.5-2.5)

output of the MAPK survival pathway in cancer cells [7]. We observed that OTX008 exposure inhibited Gal1, p-ERK1/2, p-AKT and p-S6 protein levels in a time dependent-manner in OTX008-sensitive cell lines. This is coherent with the report by Thijssen et al. that p-ERK1/2 and p-AKT signalling is inhibited by down-regulating Gal1 expression using Gal1 knockdown constructs and anginex treatment in endothelial cells [26].

The interaction of Gal1 with glycoproteins, ECM components and several membrane receptors has been described as a key mechanism responsible for the modulation of cell proliferation or tumour cell death [6,23]. Nonetheless, we show here that the antiproliferative effects of OTX008 are not due to the interruption of Gal1 binding to ECM components. However, the ability of OTX008 to interact with the Gal1/Sema3A/NRP-1 system could affect tumour proliferation, since OTX008 treatment enhanced the antiproliferative effects of Sema3A, probably through inhibition of the Gal1/NRP-1 interaction. While no changes in the antiproliferative effects of OTX008 were observed in the presence of exogenous Gal1, the enhanced invasiveness induced by Gal1 was fully inhibited by OTX008 in SQ20B cells. Our findings showed that pharmacologic or knockdown inhibition of Gal1 by OTX008 or sh-*LGALS1*-RNA resulted in decreased SQ20B invasion. Thus, Gal1 appears to have global effects on intracellular signalling events, eliciting changes in tumour cell behaviour which go beyond its direct involvement in the process of tumour cell invasion. Cellular effects of OTX008 in cancer cells are related at least in part to its downregulation of Gal1 protein and modulation of the Gal1/Sema3A/NRP-1 system. Further studies are warranted to elucidate the mechanisms by which OTX008 induces down-regulation of Gal1 protein in a time-dependent manner in OTX008-sensitive cell lines.

Several reports have shown that high Gal1 expression is associated with several poor prognostic parameters, such as advanced cancer stage, poor survival or cytotoxic drug resistance in several human cancers [10,14,15,18]. Combination therapeutic approaches are important in the future clinical development of anti-galectin strategies. Synergistic effects with OTX008 were not unexpected given that anginex treatment with carboplatin, radiotherapy or angiostatin has shown synergistic activity in inhibiting tumour proliferation [43,44]. OTX008 combination studies showed it to be synergistic with several cytotoxic and targeted therapies mainly when OTX008 was administered first. Understanding the mechanistic basis of these pharmacologic outcomes is critical for maximising the benefit of Gal1 inhibitors in the clinic. In this study we demonstrated that although OTX008 stand-alone treatment displays direct antitumour effects, OTX008 combination with conventional chemotherapy and targeted therapies agents are needed for OTX008 positioning in clinical development.

In vivo, OTX008-treated tumours showed both decreased Gal1 and Ki67 expression, consistent with its antiproliferative effects observed in cultured cells. Recent data also demonstrated that Gal1 knock-down in cancer cells could reduce tumour angiogenesis by inhibiting endothelial cell migration and proliferation in xenograft models [9,45]. In addition, we observed that microvessel diameter and VEGFR2 expression were significantly reduced in OTX008-treated tumours. These data strongly suggest that OTX008 induces tumour vasculature normalisation, as it was previously reported in MA148 human ovarian cancer xenografts and B16F10 murine melanoma model after OTX008 treatment [39]. In addition, similar findings were recently described by Croci et al. for a neutralising Gal1-specific mAb in anti-VEGF refractory tumours [46].

Following promising preclinical results with anginex [9,43,44], new generations of anti-Gal1 compounds with improved specificity were designed to reduce the therapeutic dose and minimise molecular size. The data presented here for one of them, support the validity of using the small molecule inhibitor OTX008 as a novel clinical approach to inhibit cancer cell proliferation, prevent tumour invasion, and block angiogenesis, while also improving the efficacy of several current anticancer therapies when used in combination.

Conflict of interest statement

Esteban Cvitkovic is founder and stockholder of OncoEthix. Eric Raymond is a consultant for OncoEthix.

Acknowledgements

This work was supported by Oncoethix, the Foundation Nelia & Amadeo Barleta (FNAB), and the Association d'Aide à la Recherche & à l'Enseignement en Cancérologie (AAREC). The authors thank Sylvie Mosnier (Departments of Pathology, Beaujon Hospital) for the IHC staining. Authors acknowledge the Institut des Vaisseaux et du Sang (Paris, France) for the technical support. Authors would like to thank Sarah MacKenzie for language editing (funded by AAREC).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2014.06.015.

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