Generation and characterization of rendomab-B1, a monoclonal antibody displaying potent and specific antagonism of the human endothelin B receptor

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Abbreviations: ETBR, endothelin B receptor; ETAR, endothelin A receptor

Endothelin B receptor (ETBR) is a G protein-coupled receptor able to bind equally to the three identified human endothelin peptides. It is expressed primarily on vascular endothelial cells and involved in various physiological processes including vascular tone homeostasis, enteric nervous system development, melanogenesis and angiogenesis. Furthermore, overactivation or overexpression of ETBR have been associated with the development of various diseases such as cardiovascular disorders and cancers. Therefore, ETBR appears to be relevant target for the therapy or diagnosis of highly prevalent human diseases. In this study, we report the in vitro characterization of rendomab-B1, a monoclonal antibody (mAb) obtained by genetic immunization, which selectively recognizes the native form of human ETBR (hETBR). Rendomab-B1 is the first-reported mAb that behaves as a potent antagonist of hETBR. It recognizes an original extracellular conformational epitope on the receptor, distinct from the endothelin-1 (ET-1) binding site. Rendomab-B1 not only blocks ET-1-induced calcium signaling pathway and triggers rapid receptor internalization on recombinant hETBR-expressing cells, but also exerts pharmacological activities on human vascular endothelial cells, reducing both cell viability and ET-1-induced hETBR synthesis. In addition, binding experiments using rendomab-B1 on different melanoma cell lines reveal the structural and functional heterogeneity of hETBR expressed at the surface of these cancer cells, strongly suggesting the existence of tumor-specific receptors. Collectively, our results underscore the value of rendomab-B1 for research, therapeutic and diagnostic applications dealing with hETBR.

Introduction

The endothelin family is composed of three identified isopeptides termed ET-1, ET-2 and ET-3. Each peptide is characterized by a 21-amino-acid primary sequence, two intramolecular disulfide bridges and strong sequence similarities with cardio-toxic peptides (the sarafotoxins) found in the venom of the snake Atractaspis engaddensis.1 In humans, endothelins are produced by various organs2-4 following a complex biosynthesis pathway requiring the cleavage of pro-peptides (the big-endothelins) by endothelin-converting enzymes to obtain mature and physiologically active endothelins. Among the three endothelins, ET-1 is the most abundant isoform and is mainly generated within the vascular wall. Once produced, the endothelins exert their biological action in a paracrine or autocrine fashion and intervene in a wide range of physiological functions such as vascular tone homeostasis,5-7 neural crest development,8 ovarian cycle,9 cell proliferation, angiogenesis and inflammation.10 To mediate their numerous physiological effects, endothelins activate two distinct G protein-coupled receptors: ETA receptor (ETAR) and ETB receptor (ETBR). ETBR equally binds all three endothelin isoforms, whereas ETAR shows a higher affinity for ET-1 and ET-2 than for ET-3. Both receptors present a quasi-ubiquitous expression pattern, but ETAR predominates on vascular smooth muscle cells and cardiomyocytes while ETBR is particularly abundant on vascular endothelial cells.

Endothelins and their receptors (i.e., the endothelin axis) have been implicated in a large variety of diseases.4 Vascular endothelial ETBR notably is involved in the two most prevalent diseases in humans, i.e., cardiovascular disorders and cancers.
Overexpression or overstimulation of endothelial ETBR promotes atherosclerotic lesions, tissue fibrosis and atheroma plaque development. In the field of oncology too, the pathological role of endothelial ETBR has been particularly documented in recent years since it has been reported that ETBR located in the tumor-surrounding vascular endothelium is implicated in: (1) cancer cell growth (by increasing neoangiogenesis); (2) invasiveness and metastatic dissemination (by promoting macrophage homing to tumors, which release extracellular matrix-degrading metalloproteinases); and (3) tumor escape from immune surveillance (by largely reducing cytotoxic T cell homing to tumors). Besides this deleterious role played by endothelial ETBR in any kind of cancer, it has also long been reported that some tumor cells themselves can overexpress ETBR, which contributes to their development and aggressiveness.

In this context, we decided to develop new tools not only to gather more information on human ETBR (hETBR) cytochemical distribution, structure and roles under both normal and pathological conditions, but also, ideally, to block this receptor, i.e., to exert antagonist activity when hETBR is associated with vascular disease and cancer, for example. Currently, the only way to investigate the structural-pharmacological properties of ETBR is to use small chemical agents, displaying either antagonist (bosentan, BQ-788) or agonist (IRL-1620, sarafotoxins) properties. These small molecules are very useful but they generally lack specificity (e.g., bosentan is a dual ETAR/ETBR antagonist), give relatively little information concerning receptor structure, are not adapted for imaging and often remain ineffective for some therapeutic applications. Consequently, we focused on the development of monoclonal antibodies (mAbs), which have emerged as very attractive alternatives to conventional chemical agents for tumor labeling and drug development. Monoclonal antibodies are often more specific than "small molecules," allowing discrimination between highly similar target isoforms or subtle conformational variations.

They can be used in a wide range of assays (e.g., flow cytometry, immuno-imaging, western blotting) and also for protein purification and crystallization. Finally, from a clinical point of view, mAbs present numerous advantages over other pharmaceuticals that position them among blockbuster drugs in various indications.

To this end, we produced a panel of 27 murine mAbs targeting human ETBR using an original electroporation-aided DNA immunization strategy that favors the production of pharmacologically active antibodies directed against the native form of membrane-spanning proteins. Competition binding tests, performed early during the screening process, revealed that, among these specific anti-hETBR antibodies, only one displayed very strong antagonist properties and thus appeared particularly suited to our purpose. This antibody was named rendomab-B1 and was selected for further detailed in vitro characterization, which is described here. The properties of the other anti-hETBR mAbs will be reported later. Rendomab-B1 can be distinguished from all other formerly produced anti-ETBR antibodies by its potent antagonist properties and its ability to induce rapid hETBR internalization, its capacity to trigger long-term hETBR mRNA downmodulation in endothelial cells, and finally by an original conformational epitope on hETBR. Moreover, unexpectedly, it displayed the noteworthy ability to discriminate between hETBR expressed on melanoma vs. non-cancerous cells, thus suggesting that melanoma-specific hETBR might exist. Together, these results suggest that rendomab-B1 is an outstanding biological tool, not only to block hETBR signaling pathways in the context of a therapeutic use, but also to investigate the molecular structure and role of hETBR under both normal and pathological conditions.

Results

Production and selection of rendomab-B1. As we demonstrated in a previous paper, DNA immunization is a powerful approach to generate specific polyclonal antibodies against hETBR, and more generally against GPCRs. Therefore, the same method of immunization was used in the present study to generate anti-hETBR mAbs (Fig. 1A, see legend). The two mice with the highest ratio of specific antibodies, determined by flow cytometric analysis (Fig. 1B), were sacrificed and used for hybridoma production. Hybridoma supernatants were first screened using the living cell-based ELISA that we described previously. Then, the specificity of positive hybridoma was further confirmed by flow cytometric tests (data not shown) performed on three cell types (CHO cells transfected or not with hETBR, or transfected with an irrelevant GPCR, the NK1 human receptor for the neuropeptide substance P). These differential screenings, followed by the limiting dilution step, led to the selection of 27 pure clones (out of 169 initially selected by the ELISA test). All antibodies were then isotyped (3 IgM, 8 IgG1, 9 IgG2a, 4 IgG2b and 3 IgG3), produced in large quantities and affinity-purified. Purity was checked by gel electrophoresis (not shown). Since we were particularly searching for an antibody displaying antagonist properties, for the different reasons explained above, competition binding experiments were performed at this stage, using flow cytometry. CHO-hETBR cells were incubated in the presence of a fixed ET-1 FAM concentration (10 nM), with or without a single concentration of each antibody (100 nM), including control isotypic antibodies. Under these conditions, only one antibody, which we called rendomab-B1, inhibited ET-1 binding by almost 90%, whereas the percent of binding inhibition measured for all other antibodies, if any, did not exceed 30% (not shown). As a consequence, we focused on rendomab-B1 (an IgG2b) for further in vitro characterization. The 26 other specific anti-hETBR mAbs, which displayed binding properties clearly different from those of rendomab-B1, could be also considered as valuable tools for studying hETBR and will be described elsewhere.

Rendomab-B1 binds to native hETBR on different cell lines with a high affinity, without cross-reaction with hETAR. To confirm the specificity observed during the screening procedure with hybridoma supernatants and preliminary competition experiments, we checked the ability of the purified rendomab-B1 to recognize native hETBR expressed at the surface
Using human ETAR-stably transfected CHO cells (kindly provided by Dr. M. Iglarz, Actelion, Paris, France), we then showed that rendomab-B1 did not cross-react with hETAR, despite 63% sequence identity. Finally, using ELT3 mouse cells expressing both ETAR and ETBR (received as a generous gift from Dr. Z. Tanfin), we found that rendomab-B1 did not recognize any murine ET-1 receptor (not shown). For all the above-mentioned experiments, the expression of endothelin receptors at cell surfaces was systematically checked using fluorescein-labeled ET-1 (ET-1-FAM, Fig. 2A, upper panel and data not shown).

Rendomab-B1 inhibits ET-1 binding to hETBR, behaving as a mixed non-competitive inhibitor. The interference of rendomab-B1 with endothelin binding to hETBR was evaluated in competition experiments using flow cytometry. As shown in Figure 3A, rendomab-B1 inhibits ET-1-FAM binding to hETBR and, interestingly, the inhibition potency of rendomab-B1 was almost three-fold higher than that of the reference specific hETBR inhibitor peptide, BQ788 (IC₅₀ of 22 vs. 63 nM, respectively). Conversely, endothelins (ET-1, ET-2, ET-3) are able to prevent rendomab-B1 binding with the same potency, slightly higher than that of sarafotoxin 6c (Fig. 3B). The competition between rendomab-B1 and ET-1 was also demonstrated by confocal microscopy experiments in which rendomab-B1 staining on hETR-expressing CHO cells was largely attenuated by ET-1 addition (Fig. 3C).
Rendomab-B1 exerts pharmacological effects not only by competing with ET-1 but also by triggering rapid internalization of hETBR. Since rendomab-B1 was able to antagonize hETBR with high potency, we then tested whether the antibody could modulate hETBR pharmacology in CHO cells. As illustrated in Figure 5A and B, rendomab-B1 potently antagonizes the intracellular calcium influx induced by 2 nM ET-1, and even completely blocks it at a concentration of 200 nM. Interestingly, the concentration leading to 50% loss in calcium mobilization (IC₅₀) was three times lower than that of the reference antagonist BQ788 (45 vs. 136 nM).

In these experiments, it is important to note that the inhibitors (rendomab-B1 and BQ788) were pre-incubated with cells at 4°C before the calcium influx assay, to ensure that any binding that occurred during the pre-incubation period did not affect the results. The mechanism of these mutual binding inhibitions was then investigated through flow cytometric experiments in which both rendomab-B1 and ET-1 concentrations were varied. As shown in Figure 4A, ET-1 has a negative effect on both maximal binding and apparent affinity of rendomab-B1. Scatchard analysis of these data, displaying a pattern of intersecting lines at a single point below the x axis, confirmed a mixed non-competitive mechanism (not shown). Conversely, Figure 4B shows that maximal binding and apparent affinity of ET-1 were both lowered by increasing concentrations of rendomab-B1. These results indicate that ET-1 and rendomab-B1 behave as mixed non-competitive inhibitors toward each other, and thus suggest that they recognize distinct sites on hETBR.
at 37°C, 2 h before ET-1 stimulation. As a consequence, the antagonist property of rendomab-B1 could be due, at least in part, to the induction of hETBR internalization. To address this issue, we performed confocal microscopy experiments to localize hETBR in cells, following rendomab-B1 addition and subsequent incubation at 37°C or at 4°C for 2 h. As shown in Figure 5C, the localization of rendomab-B1 staining changed depending on the incubation temperature. When rendomab-B1 was incubated for 2 h at 4°C (left panel), the staining was essentially membrane-located. Conversely, incubation at 37°C led to the appearance of numerous intracellular spots that partly colocalized with the early endosomal marker EEA1 (Fig. 5C, right panel). Therefore, these data indicate that the inhibition observed in the calcium influx assay (with a 2 h preincubation) is at least partly due to rendomab-B1-induced hETBR internalization, which desensitizes cells to ET-1.

To assess whether rendomab-B1 is able to block the ET-1-induced calcium signaling pathway independently of its capacity to induce hETBR internalization, we varied its preincubation time with cells preceding ET-1 addition (Fig. 5D). Interestingly, a preincubation time as short as one minute was sufficient to strongly inhibit the intracellular calcium release (90% loss), similarly to 2 h preincubation. This one-minute preincubation with rendomab-B1 (not sufficient to induce hETBR internalization) proves that rendomab-B1 is able to block the ET-1-induced calcium influx regardless of hETBR internalization. However, we did not observe any inhibition when rendomab-B1 and ET-1 were simultaneously added to the cells, in contrast to what is observed with BQ788. This result can be explained by the large size of the antibody compared with BQ788 (150 kDa vs. 664 Da), which prevents rendomab-B1 from reaching the receptor before ET-1 and antagonizing the signaling pathway.

Finally, we performed additional experiments to directly demonstrate the internalization of hETBR induced by rendomab-B1 in CHO-hETBR cells and to compare its efficiency to that induced by ET-1 itself. CHO-hETBR cells were pre-incubated during 2 h at 37°C in the presence of rendomab-B1, or a control antibody (same isotype as rendomab-B1), or unlabeled ET-1, or in the absence of any compound (in the buffer alone). Afterwards, cells were rinsed and kept in the presence of ET-1-FAM for 30 min at 4°C before flow cytometry analysis. As shown in Figure 5E, pre-incubations in the presence of rendomab-B1 or ET-1 considerably lowered the membrane labeling, whereas labeling observed after exposure to the control antibody remained identical to that obtained when cells were kept for 2 h in the buffer alone. This result clearly indicates that rendomab-B1 triggers hETBR internalization in CHO cells, almost as efficiently as ET-1, which could actually contribute to its antagonist effect.
two located within the N-terminal tail and one covering the main part of the second extracellular loop (Fig. 6A–C). More precisely, immunoreactive spots J21 to K10 and K22 to L11 match amino acid residues 45–69 and 70–94 of the N-terminal domain, whereas spots N11 to N13 and O6 to O13 correspond to amino acids 251–277 of the E2 loop. Interestingly, the most intensely stained consecutive spot series (O6 to O13), which encompassed residues 259-PVQ KTÅ FMQ FYK TAK DWW L277, showed a correlation between the staining intensity and the appearance of the “KTÅ” sequence, with a peak signal (spot O9) when the two motifs “KTÅ” and “KTÅK” were present in the dodecapeptide (Fig. 6D). Thus, the peptide sequence corresponding to the spot O9 and especially the “KTÅ” motif seems to be a major determinant for rendomab-B1 binding. More importantly, the fact that rendomab-B1 interacts with three linear regions located within distinct extracellular domains (N-terminal tail and E2 loop), without any sequence similarity, suggests that rendomab-B1 might recognize a conformational epitope. This assumption is reinforced by binding experiments showing that rendomab-B1 affinity for hETBR is strongly impaired when cells overexpressing the receptor are fixed by 4% paraformaldehyde, by the fact that rendomab-B1 proved to be ineffective in probing hETBR in western blot tests and finally by the observation that the antibody was unable, under our experimental conditions, to bind any epitope peptide that we synthesized and coated alone or in combination on a 96-well plate (data not shown).

Rendomab-B1 triggers internalization of hETBR in HUVECs, reduces cell viability and leads to hETBR mRNA downmodulation. In humans, ETBR is mainly expressed by vascular endothelial cells and activated by ET-1 in an autocrine fashion. In this context, we tested whether rendomab-B1 was able to bind efficiently to hETBR expressed by human umbilical endothelial cells (HUVECs). As illustrated in Figure 7A, rendomab-B1 significantly stained HUVECs, a staining completely inhibited when cells were pre-incubated with sarafotoxin 6c, a specific ligand of hETBR. It is worth noting that rendomab-B1 staining on HUVECs was rather weak, probably due to the high amounts of endothelins secreted by these cells, which we estimated as ~100 pM/million cells through a sensitive endothelin ELISA (data not shown). Once bound to membrane receptor, rendomab-B1 was able to trigger hETBR internalization in HUVECs as efficiently as ET-1 itself (Fig. 7B), just as it did in CHO-hETBR cells (Fig. 5E).

ET-1 has been described as a pro-survival and mitogenic factor for endothelial cells and, as mentioned above, pathophysiological roles of hETBR, namely in cardiovascular diseases and cancers, are frequently linked to its over-stimulation or overexpression in the vascular endothelium. Accordingly, we tested whether rendomab-B1 was able to affect HUVEC proliferation/survival by incubating cells with 500 nM rendomab-B1 (or BQ788) for several days, before assessing cell viability using the

Together, these findings demonstrate that rendomab-B1 is able to completely block the ET-1-induced calcium signaling pathway by directly competing with ET-1 binding to hETBR and by triggering rapid hETBR endocytosis leading to cell desensitization to ET-1 stimulation.

Rendomab-B1 recognizes a discontinuous epitope composed of three extracellular sequences of hETBR. To gain more insight into the rendomab-B1 binding site on hETBR, we undertook an epitope mapping analysis using a set of 147 overlapping dodecapeptides, frameshifted by one amino acid, and corresponding to the hETBR three extracellular loops (E1, E2 and E3) and N-terminal tail. Incubation of the membrane with rendomab-B1 revealed three discontinuous linear immunoreactive regions:

**Figure 4.** Rendomab-B1 behaves like a non-competitive inhibitor of ET-1 toward hETBR. (A and B) Both ET-1 and rendomab-B1 concentrations were varied and added simultaneously to CHO-hETBR cells. After 24 h incubation at 4°C, rendomab-B1 binding (using FITC-labeled antibody) in the presence of increasing concentrations of unlabelled ET-1, or ET-1 binding (using FAM-labeled peptide) in the presence of unlabelled rendomab-B1, were measured by flow cytometry. Resulting binding curves are presented in (A) and (B), respectively. Data were fitted using GraphPad Prism software. The experiments were repeated three times.
MTT dye. As illustrated in Figure 7C, rendomab-B1 induced a sharp decrease in cell viability (65% loss), here again being more potent than BQ788 (20% loss). A similar result was obtained using an anti-endothelin-1 mAb, confirming the autocrine survival role of ET-1 in HUVECs. Since rendomab-B1 induces hETBR internalization in HUVECs (Fig. 7B), it is possible that the observed decrease in cell viability could be due, at least in part, to this desensitization process.

Finally, we sought to know whether rendomab-B1 could additionally lead to a downmodulation of hETBR expression in HUVECs. As shown in Figure 7D, exposure of HUVECs to rendomab-B1 for 24 h not only completely blocked ET-1-induced hETBR mRNA upregulation (as does BQ 788), but also resulted in an additional 25% reduction in the quantity of hETBR mRNA compared with untreated control cells, this additional decrease not being observed with BQ788.

**Rendomab-B1 affinity for hETBR is greatly impaired in melanoma cell lines.** As mentioned above, some tumor cells themselves, like melanoma, are known to overexpress hETBR, which favors their proliferation or survival.17 Accordingly, rendomab-B1 binding was tested on three human melanoma cell lines: UACC257, SLM8 and WM266–4. Amazingly, curves of rendomab-B1 binding to all these melanoma cells displayed very low signals and did not present any plateau (Fig. 8F), unlike those obtained with hETBR transfected cells (Fig. 8E), which reflects a poor affinity for melanoma and renders apparent affinity calculations imprecise. This result is all the more surprising because we checked that ET-1-FAM labels the three cell lines in a saturable fashion (Fig. 8B), with similar affinities to those obtained with hETBR transfected cell lines (Fig. 8A), clearly demonstrating the presence of functional endothelin receptors at the surface of melanoma cell lines. To ensure that ET-1-FAM binding was indeed due to hETBR expression, we confirmed that the signals obtained were inhibited by BQ788 and sarafotoxin 6c (specific hETBR antagonist and agonist, respectively), but not by FR139317, an hETAR specific antagonist (data not shown).
broad range of physiological functions and in some of the most prevalent human disorders, as reflected by the fact that about one third of all marketed therapeutics act on GPCRs.33,34 Despite their relevant role in various human diseases, no GPCR-targeting therapeutic antibodies have been approved so far. In this study, we describe the production and the characterization of a new mAb targeting and potently inhibiting a therapeutically relevant GPCR, the human endothelin B receptor, which has been implicated in several diseases, including cardiovascular diseases and cancers.

The generation of pharmacologically active antibodies recognizing the native conformation of GPCRs is not an easy task, and peptide immunizations using GPCR extracellular fragments or purified receptors sometimes result in antibodies unable to bind the receptor in its membrane context, as emphasized in a recent review.24 To bypass this major problem, which arises from the difficulty of producing large quantities of stable and correctly folded GPCRs, we used an electroporation-aided DNA immunization approach involving in vivo immunogen production and folding. As previously described by different groups27,28,35,36 including ours,29,30 this technique has been successfully used to generate polyclonal and monoclonal antibodies against the native conformation of GPCRs. The advantage of this technique is once more illustrated in the present study, since we obtained 27 mAbs recognizing native hETBR. The fact, however, that only one antibody, rendomab-B1, was able to potently antagonize the receptor emphasizes that obtaining a pharmacologically active antibody still remains a difficult technical challenge.

Finally, the hypothesis of a possible competition between rendomab-B1 and endothelins secreted by melanoma cells, which could explain an absence of significant antibody binding, was very unlikely since no endothelin-like immunoreactivity was detected in cell culture medium using a picomolar-range sensitive endothelin ELISA (data not shown). To clarify this unexpected rendomab-B1 behavior on melanoma cells, we used the anti-hETBR polyclonal antiserum generated during the immunization procedure. As shown in Figure 8C and D, anti-hETBR polyclonal antibodies recognized both CHO-hETBR cells and melanoma cells similarly. This indicates that the specificity of rendomab-B1 is very peculiar and clearly not representative of the specificities of most anti-hETBR antibodies present in the antiserum. It is also worth noting that polyclonal antisera saturating signals (B_{MAX}) obtained with hETBR-transfected cells and melanoma were similar (Fig. 8C and D), whereas corresponding saturating signals obtained with ET-1-FAM were very different (Fig. 8A and B), which suggests a functional or a structural heterogeneity of hETBR at the surface of melanoma, only a small proportion of receptors in these tumor cells being able to bind ET-1.

**Discussion**

G protein-coupled receptors (GPCRs) constitute the largest family of cell surface signaling proteins, with approximately 900 members, accounting for more than 2% of the genes identified in the human genome.35 These receptors have a pivotal role in a broad range of physiological functions and in some of the most prevalent human disorders, as reflected by the fact that about one third of all marketed therapeutics act on GPCRs.33,34 Despite their relevant role in various human diseases, no GPCR-targeting therapeutic antibodies have been approved so far. In this study, we describe the production and the characterization of a new mAb targeting and potently inhibiting a therapeutically relevant GPCR, the human endothelin B receptor, which has been implicated in several diseases, including cardiovascular diseases and cancers.

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**Figure 6.** Rendomab-B1 recognizes a discontinuous epitope on hETBR, involving N-terminal tail and E2 loop regions. (A) Rendomab-B1 epitope was investigated using a Peptide membrane spotted with dodecapeptides (frameshifted by one amino acid) covering the entire hETBR extracellular regions. Interacting peptides were revealed using an alkaline phosphatase conjugated secondary antibody. (B) Schematic localization of the regions recognized by rendomab-B1 on hETBR. (C) Spot signals obtained on the membrane were quantified by densitometry. Isolated (no more than two consecutive) spots, as well spot series not presenting any signal peak higher than three times the background, are not represented and were considered as irrelevant. (D) Table mentioning the sequence of most intensely stained spots on the membrane. Underlined motifs correspond to putative key residues for rendomab-B1 binding. This experiment was done twice with two independent membranes. An isotype control antibody did not generate any staining.
pharmacological assays revealed that rendomab-B1 was a potent hETBR antagonist (once again three times better than BQ788) able to silence completely the ET-1-induced IP3-calcium signaling pathway. Since it has been very recently reported\(^3\) that, upon ETBR binding, ET-1 can trigger additional signaling pathways (e.g., β-arrestin-1 and c-Src activation), it would be interesting to investigate whether rendomab-B1 could also antagonize these alternative pathways.

To further investigate the inhibitory mechanism of rendomab-B1, we undertook competitive binding experiments, as frequently

Experiments using flow cytometry and hETBR stably transfected cell lines demonstrated that rendomab-B1 was highly specific for human ETBR and did not cross-react with hETAR, the second endothelin receptor subtype identified in mammals. This restrictive specificity is an interesting feature that supports the use of mAbs to selectively target GPCRs without interfering with other family members, a problem often encountered with small molecules directed against GPCRs.\(^2,^3\) The apparent affinity of rendomab-B1 for ETBR was found to be remarkably high (around 600 pM) which is a favorable point for future possible diagnostic or therapeutic applications. More importantly, we demonstrated that rendomab-B1 competed with ET-1 binding to hETBR with almost three times greater efficacy than BQ788, the reference ETBR specific inhibitory compound. Moreover,
recognizes a conformational epitope that might arise from the interaction between the N-terminal tail and the E2 loop in the folded hETB receptor, as already suggested for other mAbs recognizing different noncontiguous regions on their respective membrane targets.\textsuperscript{40,41} Collectively, these findings highlight the value of rendomab-B1 in investigating hETBR structure and in gathering more information about the location of the endothelin binding site, whose precise delineation is still unclear despite several recent publications tackling the issue.\textsuperscript{42,43} Furthermore, this original non-competitive inhibitory mechanism, rarely found for small inhibitory molecules like BQ788 that generally compete with natural ligands for the receptor orthosteric site, underlines the value of developing therapeutic antibodies that offer the possibility of targeting original epitopes on GPCRs, potentially linked to new pharmacological activities and better receptor selectivity.\textsuperscript{22}

To our knowledge, this is the first time that an antagonist mAb targeting hETBR has been described. Several groups have already reported the production of ETBR-specific mAbs,\textsuperscript{44-46} but none of them showed any direct pharmacological activity on this receptor. Kondoh et al.\textsuperscript{44} were the first to produce ETBR- (and also ETAR-) specific mAbs using rat lung membranes as immunogen; however, no proof of ETBR cell surface labeling was mentioned. More than 10 y after, Yamaguchi et al.\textsuperscript{45} reported the production of five mAbs recognizing hETBR in its membrane context. Nevertheless, the authors did not comment on any competition with natural ligands. Finally, a very recent publication by Asundi et al.\textsuperscript{46} nicely presents the production and characterization of two mAbs specifically targeting hETBR. These antibodies, obtained through two independent cell fusions after different immunization protocols, recognized both recombinant hETBR and several melanoma cell lines, unlike rendomab-B1. None of them, however, was able to compete efficiently with ET-1 and thus remained without any pharmacological activity. Together, these observations clearly show that rendomab-B1 is a unique molecular tool, harboring original and unprecedented pharmacological properties in the field of anti-ETBR mAbs.

used to characterize the mechanism of enzyme inhibitors.\textsuperscript{39} Our results demonstrate that rendomab-B1 behaves as a non-competitive inhibitor, implying that it possibly recognizes an extracellular region of hETB that is structurally distinct from, but functionally related to, the ET-1 binding site. In fact, using a Pepscan analysis, rendomab-B1 epitope was mapped to three discontinuous hETBR extracellular regions, located within the N-terminal tail and the E2 loop. This indicates that rendomab-B1 actually

Figure 8. Rendomab-B1 poorly recognizes hETBR expressed by human melanoma cell lines. (A and B) hETBR transfected cells and human melanoma cells (UACC257, SLM8, WM266–4) were incubated with varying concentrations of ET-1 FAM for 24 h and analyzed by flow cytometry. (C and D) Cells were stained with varying concentrations of anti-hETBR polyclonal sera before flow cytometry analysis. (E and F) Cells were stained with varying dilutions of rendomab-B1 and resulting fluorescence was measured by flow cytometry. All binding curves (MFI vs. concentrations) were analyzed with GraphPad Prism to collect binding parameters.
In pathological conditions, ETBR overexpression or over-stimulation in the tumor-surrounding vascular endothelium, and more generally in stromal cells near tumors, has been involved in pivotal steps of cancer progression including neovascularization, immune system escape and metastatic spread. Since multiple cancers cells overexpress ET-1, a deleterious paracrine loop promoting tumorigenic effects is thus established. In this context it is very interesting that, at least in HUVECs used here as a model of endothelial cells, rendomab-B1 appears not only to antagonize the ET-1/hETBR-mediated effect (as reflected by the large drop in HUVEC viability when exposed to rendomab-B1), but also to completely block ET-1-induced hETBR expression (as reflected by a large reduction in hETBR mRNA in endothelial cells incubated in the presence of rendomab-B1), both effects being possibly explained, at least in part, by hETBR internalization triggered by this mAb. Since preliminary results indicate that rendomab-B1 is also able to recognize other endothelial cells naturally expressing hETBR (e.g., immortalized human cerebral endothelial cells hCMEC/D3 and vascular endothelial cells in patients suffering from low grade-glioma; Drs. P.O. Couraud, Institut Cochin, and J.P. Hugnot, INSERM, Institute of Neuroscience, respectively, unpublished observations), the present data could be considered together as a proof of principle for the use of rendomab-B1 for breaking the vascular hETBR-mediated tumor-promoting vicious circle described above, hopefully with greater efficiency than BQ-788. These results are also promising in the context of other diseases involving the dysregulation of hETBR in the vascular wall. For instance, rendomab-B1 could serve as a diagnostic tool for imaging endothelial hETBR in some vascular diseases in humans (Dr. L. Mouthon, Hôpital Cochin, unpublished observations), and perhaps as a therapeutic tool whenever endothelin receptors have to be efficiently blocked. Of course, such a potential use would require chimerization or humanization of rendomab-B1 and the further analysis of the binding properties of these derived compounds.

Finally, the unexpected observation that rendomab-B1 poorly recognizes hETBR on melanoma cell lines strongly suggests that hETBR expressed by these latter cells presents tumor-specific structural characteristics. The poor affinity of rendomab-B1 for hETBR in a tumor context could be explained by the N-terminal proteolysis of hETBR. Indeed, hETBR has been shown to undergo a ligand-induced metalloproteinase cleavage at R64-S65 in transfected HEK and vascular smooth muscle cells. Under our experimental conditions, no endothelin secretion by melanoma cells vs. non-cancerous cells. This very interesting finding emphasizes the value of rendomab-B1 in the detection and further characterization of the structural features of melanoma-expressed hETBR, but also paves the way for the development of mAbs specifically recognizing the “melanoma-specific” isoform(s) of hETBR. Therefore, rendomab-B1 appears as a very attractive molecular tool to antagonize pathological hETBR signaling, and to investigate hETBR molecular structure, distribution and roles in both human health and diseases.

**Materials and Methods**

**Animals.** Six-week-old female C57BL/6 mice from Janvier were kept in a specific pathogen-free animal facility. All animal
experiments complied with French animal experimentation regulations.

**Plasmids.** The cDNA clone of the human ETBR (a generous gift from Dr. M.J. Brownstein) was subcloned in pcDNA3.1 vector (Invitrogen). The integrity of the construct was confirmed by sequence analysis. Silencer select siRNAs targeting human ETBR were from Ambion. Dr. P. Robin kindly provided the plasmid containing the cDNA of the rat ETBR.

**Transfection experiments and cell cultures.** CHO cells (ECACC) were cultured in Ham-F12 medium, HEK293T (EACC) in DMEM medium and human umbilical vascular endothelial cells (HUVECs; Lonza) in EBM-2 medium containing endothelial cell growth supplements. Melanoma cell line SLM-8 was a kind gift from Dr M. Vigier, Hôpital Saint Louis), UACC257 and WM266–4 cell lines were from NCI-60 and ECACC, respectively. All melanoma cell lines were cultured in DMEM/F-12 medium except for UACC257, which was grown in RPMI1640. All media were supplemented with 10% fetal calf serum, 1 mM pyruvate, 1% nonessential amino acids, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. All media and cell culture supplements were from Invitrogen. The cDNA for ETBR was transfected and stably overexpressed in CHO and HEK cells using FUGENE HD reagent (Roche Diagnostics). Human ETAR stably transfected CHO cells were kindly provided by Dr. M. Iglarz (Actelion). Stably transfected cells with human ETBR or ETAR are termed CHO-hETBR, CHO-hETAR or HEK-hETBR.

**DNA immunization protocol and production of monoclonal antibodies.** DNA immunizations were done according to a previously described protocol.²⁹ For mAb production, the two mice presenting the highest amount of specific antibody received three final cellular boosts with HEK-hETBR cells (5 × 10⁶ cells in 200 μL i.p., one boost per day) 3 d prior to sacrifice. Collected splenocytes were fused to NS1 mouse myeloma cells as previously described.³⁶ Hybridoma supernatants were screened for production of anti-hETBR specific antibodies by a living cell-based ELISA test,²⁹ using both untransfected and CHO-hETBR cells as targets. Specificity and reactivity of antibodies were further confirmed by flow cytometry. All hybridomas were subcloned by limiting dilutions. Antibodies were isotype-d using a mouse immunoglobulin isotyping kit according to the manufacturer’s (Pierce) instructions and purified by affinity chromatography on Protein A-Sepharose (Millipore).

**Flow cytometric analysis.** Rendomab-B1 specificity and cross-reactions. For the determination of hETBR specific antibody binding, flow cytometric analysis was performed on seven cell culture models: wild-type CHO, HEK and HUVEC cell lines and stably transfected cell lines (CHO or HEK) with either hETAR or hETBR. Confluent cells in 75-cm² flasks were washed with phosphate buffer saline (D-PBS, Invitrogen) and collected after incubation at 37°C for 15 min with 3 mL of versene buffer (D-PBS/8 mM EDTA). Cells were then seeded (100,000 cells/well) onto V-shaped 96-well plates (Greiner Bio One). Plates were centrifuged, supernatant was discarded and cells were incubated for 2 h at 4°C with 100 μL of D-PBS/0.1% BSA/5% normal goat serum (NGS, Invitrogen) containing 50 nM rendomab-B1. After two washes with 150 μL of ice-cold D-PBS/0.1% BSA/1% NGS, cells were incubated for 1 h at 4°C in the dark with R-phycocerythrin (R-PE)-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch). Cells were washed twice again and resuspended in 100 μL of D-PBS/0.1% BSA. The fluorescence was finally assayed using a GUAVA flow cytometer (Guava Easycyte Plus, Millipore) and mean fluorescence intensity (MFI) of samples was measured. To control hETBR expression at cell surfaces, fluorescein-labeled ET-1 (ET-1 FAM, Phoenix Pharmaceuticals) was used. In some experiments, cells (melanoma cell lines) were pre-incubated with the specific ETAR antagonist FR139317 (Tocris) to exclusively measure ET-1 FAM binding on hETBR.

**Affinity determination and competition experiments.** For affinity measurements, saturation binding experiments were performed with increasing concentrations of rendomab-B1. Concerning competition tests, CHO-hETBR cells were incubated either with (1) fixed FITC-labeled rendomab-B1 concentration (3 nM) and varying concentrations of competitor peptides ET-1, ET-2, ET-3 or sarafotoxin 6c (Phoenix Pharmaceuticals) or with (2) fixed ET-1 FAM concentration (10 nM) and varying concentrations of rendomab-B1 or BQ788 (Phoenix Pharmaceuticals). For experiments investigating the rendomab-B1 competition mechanism, cells were incubated with varying concentrations of both rendomab-B1 and ET-1. Experiments were done at 4°C to avoid receptor internalization and overnight incubation permitted equilibrium to be reached.

**Confocal microscopy analysis.** Confocal microscopy analysis was performed on two cell culture models: the CHO-hETBR and the HEK-hETBR cells, following the method described previously.²⁹ For competition experiments, 50 nM rendomab-B1 was incubated simultaneously with 1 μM ET-1. For internalization experiments, unfixed cells were incubated at 4°C for 1 h with 100 nM of Dylight-488-labeled rendomab-B1, washed twice with D-PBS, and either kept at 4°C or returned to 37°C for 2 h before a fixation step with 4% PFA. Cells were next permeabilized with 0.5% saponin (Sigma) to stain the endosomal compartment, using anti-EEA1 antibody (Millipore).

**Calcium influx assays.** For calcium influx, 35,000 CHO-hETBR cells were plated on 96-well black-sided plates with a clear bottom and incubated at 37°C, 5% CO₂. After 24 h, medium was aspirated and cells were loaded with a calcium-sensitive dye for 1 h at 37°C using the Fluo8 NW assay kit recommendations (AAT Bioquest). Inhibitors (rendomab-B1, BQ788, isotype control antibody) were added 1 h before dye loading. The plate was then loaded onto the FLEXStation 2 multi-mode microplate reader (Molecular Devices) to obtain real-time monitoring of the calcium mobilization upon addition of 2 nM ET-1.

**Viability assay in endothelial cells (HUVEC).** 2,500 HUVECs were seeded in a 96-well plate and incubated for 24 h at 37°C, 5% CO₂. Cells were washed twice with D-PBS and incubated for 4 d in a reduced serum medium containing 500 nM rendomab-B1, isotype control antibody or BQ788. At the end of the incubation period, cells were observed with a microscope and viability was assayed with the MTT tetrazolium salt...
(Sigma): cells were incubated with 0.5 mg/mL of MTT for 1 or 2 h and then resuspended with 100 μL of DMSO. Viability was then determined by measuring the absorbance at 570 nm with a spectrophotometer.

**Peptide synthesis and hETBR-binding epitope mapping.** Overlapping 12-mer peptides, framedshifted by one residue, covering the entire extracellular amino acid sequence of the ETBR receptor, were synthesized on a cellulose membrane using the SPOT technique. Rendomab-B1 epitope mapping was performed according to a protocol described previously. Briefly, the membrane was blocked overnight, washed three times in TBS containing 0.1% (v/v) Tween (TBST) and then incubated with TBST containing 5 μg/mL rendomab-B1 for 90 min at 37°C. After three washes with TBST (pH 7.0), the membrane was stirred in a solution of anti-mouse IgG labeled with alkaline phosphatase (Sigma-Aldrich) for 1 h, washed three times again and finally incubated with the alkaline phosphatase substrate until the signal revealing the interacting peptides was sufficient. ImageJ software was used to quantify the signal obtained for each spot. Peptides were considered as antigenically relevant if they were part of a consecutive series of reactive spots, presenting at least one signal peak three times higher than the background.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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