High expression of peptide receptors as a novel target in gastrointestinal stromal tumours

Jean Claude Reubi[1], Meike Körner[1], Beatrice Waser[1], Luca Mazzucchelli[1, 2], Louis Guillou[3]

[1] Division of Cell Biology and Experimental Cancer Research, Institute of Pathology, University of Berne, Berne, Switzerland
[2] Institute of Pathology, Locarno, Switzerland
[3] Institute of Pathology, University of Lausanne, Lausanne, Switzerland

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Abstract. Recent significant advances in understanding the biology of gastrointestinal stromal tumours (GIST) have led to the introduction of a new targeted therapy (imatinib mesylate, Glivec). Hopes of a new era of a specific cancer therapy, however, have been tempered by the recognition that a significant proportion of patients who initially respond to the drug eventually become resistant to it. Given the successful development of peptide receptor scintigraphy and radiotherapy for neuroendocrine tumours, we postulated that a similar approach could offer a valid alternative in the diagnosis and therapy of GIST. Using in vitro receptor autoradiography to measure peptide receptors, we found that 16/19 GIST expressed bombesin subtype 2 receptors, 16/19 expressed vasoactive intestinal peptide subtype 2 receptors ($VPAC_2$) and 12/19 expressed cholecystokinin subtype 2 receptors, in most cases in extremely high densities. All GIST metastases were shown to express two or more of these peptide receptors in very high density. Receptors were also expressed in non-responders to Glivec or after chemoembolisation. Conversely, somatostatin subtype 2, cholecystokinin subtype 1, bombesin subtype 1 and 3, and neuropeptide Y subtype Y_1 and Y_2 receptors were not or only rarely expressed. These data represent a strong molecular basis for the use of radiolabelled bombesin, vasoactive intestinal peptide and/or cholecystokinin analogues as targeting agents to localise GIST tumours in patients by in vivo scintigraphy and/or to perform targeted radiotherapy to destroy GIST primaries, metastases and recurrences, including those resistant to Glivec.

Jean Claude Reubi (✉)
Division of Cell Biology and Experimental Cancer Research,
Institute of Pathology, University of Berne, Murtenstrasse 31,
PO Box 62, 3010 Berne, Switzerland
e-mail: reubi@pathology.unibe.ch
Tel.: +41-31-6323242, Fax: +41-31-6328999

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Introduction

Gastrointestinal stromal tumours (GIST) have gained increasing interest in the past few years. First, the interstitial cell of Cajal, an intestinal pacemaker cell, has been suggested as the cell of origin of these tumours [1]. Second, the recent recognition that most GIST have a gain-of-function mutation in *KIT* proto-oncogene, resulting in ligand-independent activation of the KIT receptor tyrosine kinase, sheds more light on their pathogenesis [2, 3]. Finally, a small molecule (STI 571, imatinib mesylate, Glivec) used for the treatment of chronic myeloid leukaemia proved to be therapeutically effective in metastatic GIST [4, 5] through selective inhibition of the enzymatic activity of the KIT tyrosine kinase pathway. This latter major clinical advance, however, has been hampered by the recognition that not all patients respond adequately to Glivec, and that many of those who initially respond may become resistant to the treatment after some time [6, 7, 8, 9]. Hence, the identification of alternative therapy modalities for GIST will represent a major challenge for future clinical investigations. In this context, it will also be very important to develop diagnostic tools able to detect small metastases and local recurrences of GIST, thereby allowing treatment of the disease at an early stage.

In recent years, it has been shown that some human cancers can overexpress specific peptide receptors and that these can be targeted for either diagnostic or radiotherapeutic purposes [10]. The best evidence has been provided for somatostatin receptors expressed in neuro-

endocrine tumours, which can currently be targeted with ^{111}In-DTPA-octreotide for their in vivo localisation or with ^{90}Y-DOTATOC for targeted radiotherapy [11]. Indeed, somatostatin receptor scintigraphy was shown to be the diagnostic tool of first choice for a subgroup of gut neuroendocrine tumours, as it was superior to all other conventional imaging methods [12], and radiotherapy with ^{90}Y-DOTATOC appears extremely promising in tumours expressing somatostatin receptors, with more than 25% remissions and about 60% disease stabilisation [13, 14, 15, 16]. More recently, other peptide receptors have emerged as being overexpressed in selected tumours [10] and appear to have a strong in vivo targeting potential. These are bombesin receptors of the BB_2 subtype, better known as gastrin-releasing peptide (GRP) receptors, which are overexpressed in prostate and breast cancers [10] and can be visualised in vivo in these tumours [17, 18]. Also cholecystokinin 2 (CCK_2) receptors expressed in medullary thyroid carcinomas (MTC) [10] can be selectively targeted in vivo [19, 20].

Peptide receptors can also be expressed in normal tissues. Of particular interest in relation to GIST is the fact that somatostatin receptors, vasoactive intestinal peptide (VIP) receptors, GRP receptors and substance P receptors of the NK_1 subtype were found to be expressed in the putative precursor cell of GIST, namely the Cajal cells of the gastrointestinal tract in animals as well as in humans [21, 22]. In the present study we therefore investigated a number of peptide receptors, including the four mentioned above, for their expression in GIST using in vitro receptor autoradiography.

Materials and methods

The peptide receptors investigated in this study include bombesin receptors [with their three subtypes, namely BB_1 (NMB receptors), BB_2 (GRP receptors) and BB_3], VIP receptors ($VPAC_1$ and $VPAC_2$ subtypes), CCK_1 and CCK_2 receptors, somatostatin receptors (sst_2 receptors), substance P receptors (NK_1 receptor subtype) and neuropeptide Y (NPY) receptors (Y_1 and Y_2 subtypes). The methodology used is in vitro receptor autoradiography identifying the respective receptor proteins morphologically as specific binding sites. The methods used for the identification of the various receptors and their subtypes were reported in detail previously [23, 24, 25, 26, 27, 28]. Subtype-selective VIP receptor autoradiography was performed using ^{125}I-VIP (2,000 Ci/mmol, Anawa, Wangen, Switzerland) as radioligand with the $VPAC_1$-selective [K^{15}, R^{16}, L^{27}]-VIP(1-7)GRP(8-27) and $VPAC_2$-selective Ro25-1553 [25]. Subtype-selective CCK receptor autoradiography was performed using ^{125}I-[D-Tyr-Gly, Nle^{28,31}]-CCK26-33 (^{125}I-CCK; 2,000 Ci/mmol, Anawa, Wangen, Switzerland) as radioligand, displaced with CCK-8 or gastrin to discriminate between CCK_1 and CCK_2 receptors [24]. Subtype-selective bombesin receptor autoradiography was performed using ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14) (2,000 Ci/mmol, Anawa, Wangen, Switzerland) as radioligand, with unlabelled GRP, NMB and [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14) to discriminate between GRP, NMB and BB_3 receptors [27]. sst_2 receptors were detected with ^{125}I-[Tyr^3]-octreotide (2,000 Ci/mmol, Anawa, Wangen, Switzerland) displaced with the sst_2-selective ligand L-779-976 [28]. NK_1 receptors were identified with ^{125}I-Bolton-Hunter-SP (2,000 Ci/mmol, Anawa, Wangen, Switzerland) as radioligand and displaced with the NK_1-selective agonist [Sar^9, Met(O$_2$)^{11}]-SP [23]. NPY receptors were detected with ^{125}I-PYY (2,000 Ci/mmol, Anawa, Wangen, Switzerland), using [Leu^{31}, Pro^{34}]-NPY as displacer for Y_1 receptors and PYY(3-36) as displacer for Y_2 recep-

Table 1. Clinicopathologic data on 15 gastrointestinal stromal tumour primaries

Site	Case	Sex	Age (yrs)	Tumour size (cm)	Mitotic index[a]	Tumour necrosis	Tumour grade: NIH Consensus Conference[b]
a) Stomach	1	M	67	6	5	No	Intermediate risk
	2	F	48	7	6	Yes	High risk
	3	F	71	5	16	No	High risk
	4	M	42	8.5	2	No	Intermediate risk
	5	M	59	9	3	No	Intermediate risk
	6	M	69	4	1	No	Very low risk
	7	F	54	0.9	1	No	Very low risk
	8[c]	F	49	20	54	Yes	High risk
b) Small intestine	9	M	71	3	1	Yes	Low risk
	10	F	78	5	1	Yes	Low risk
	11[c]	F	49	4	3	No	Low risk
c) Mesentery/epiploon	12	M	69	12	21	No	High risk
	13	M	41	20	76	Yes	High risk
	14[c]	F	67	21	2	No	High risk
	15[c]	M	82	23	33	Yes	High risk

[a] Number of mitoses/50 hpf (one high-power field: 0.174 mm^2)
[b] Reference no. [7]
[c] Metastases at diagnosis

Table 2. Receptor data on 19 gastrointestinal stromal tumour primaries (presented in the same order as in Table 1) and metastases

Site	Case	Sex	Age (yrs)	Receptors							
				Bombesin		$VPAC_2$	CCK		NPY[a]	sst_2	NK_1
				GRP	NMB		CCK_1	CCK_2			
1. Primaries											
a) Stomach	1	M	67	1,994 het		4,723	–	13,260	–	347 het	–
	2	F	48	3,002 het		–	–	–	–	–	–
	3	F	71	–		5,501	–	11,464	–	–	–
	4	M	42	2,085	12,316	1,004	1,653	12,512	–	–	–
	5	M	59	1,304 het		10,880	–	11,632	166 (Y1)	–	–
	6	M	69	1,884 het		778	7,478	5,363	827 (Y2)	492	1,212 het
	7	F	54	–		478 het	–	–	952 (Y1)	–	–
	8[b]	F	49	160 het		650	–	12,962	–	–	–
b) Small intestine	9	M	71	16,827 het		–	–	11,959 het	–	–	–
	10	F	78	31,533		6,265	–	–	–	–	–
	11[b]	F	49	3,933		1,849	–	534 het	8,013 het (Y2)	129 het	–
c) Mesentery/epiploon	12	M	69	–		705	–	–	–	1,045 het	203
	13	M	41	7,171 het		2,538	–	774	–	–	–
	14[b]	F	67	192 het		–	–	–	–	–	–
	15[b]	M	82	24,467		522	–	–	–	–	–
2. Metastases											
a) Lymph node	16	F	73	3,640		7,079	–	12,020	–	–	–
b) Liver	17[c]	M	42	18,184		5,787	–	747	–	–	1,663 het
	18	F	21	1,142 het		3,698	–	10,459	2,598 (Y1)	–	940
c) Peritoneum	19[d]	F	41	8,329		5,242	6,826	–	–	–	–

Numbers represent receptor density in each tumour expressed as dpm/mg tissue
–, Receptor negative; het, heterogeneous receptor distribution
[a] Y1 and Y2 indicate the predominant NPY receptor subtype present in the tumour
[b] Metastases at diagnosis
[c] Chemo-embolisation prior to sampling
[d] Glivec therapy prior to sampling

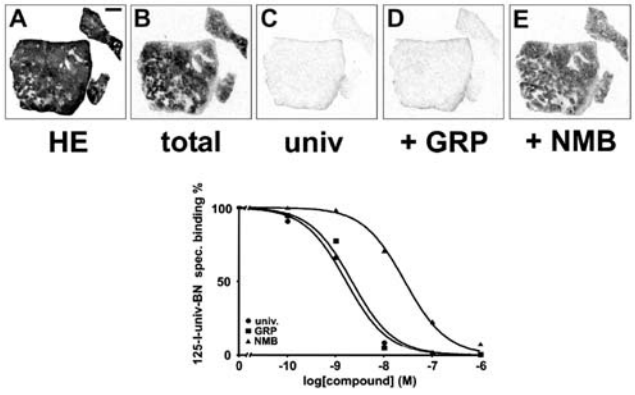

Fig. 1A–E. Characterisation of GRP receptors (BB_2) in GIST. **A** Haematoxylin-eosin stained section. *Bar*=1 mm. **B** Autoradiogram showing total binding of ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-BN(6-14). **C** Autoradiogram showing non-specific binding in the presence of 50 n*M* of unlabelled [D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14), as universal ligand. **D** Autoradiogram showing ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-BN(6-14) binding in the presence of 50 n*M* GRP. Full displacement is observed. **E** Autoradiogram showing ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-BN(6-14) binding in the presence of 50 n*M* NMB. Only weak displacement is seen. *Bottom part*: Complete displacement curves in a GRP receptor-expressing GIST. ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-BN(6-14) is displaced by nanomolar concentrations of the unlabelled analogue (●). Moreover, GRP (■) displaces the radioligand with high affinity while NMB (▲) is much less active

tors [26]. In all experiments, the autoradiograms were quantified using a computer-assisted image processing system, as described previously [25, 26]. Radiolabelled tissue sections were exposed to ^{3}H-Hyperfilms together with standards (Autoradiographic [^{125}]microscales, Amersham) that contained known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentration. The image analyser was calibrated to the standards; it performed interpolation to read values that lay between those of the film standards. A tumour was considered as receptor-positive when the optical density measured over a tissue area in the total binding section was at least twice that of the non-specific binding section. In the present study, 19 frozen GIST samples were analysed (Tables 1, 2). The clinical data of the patients and the tumour characteristics are listed in Table 1.

Results

All GIST tested in this study expressed peptide receptors. Three of these peptide receptors, namely GRP (BB_2) receptors, $VPAC_2$ receptors and CCK_2 receptors, were found with a very high incidence in these tumours (Table 2). GRP receptors were found in 16/19 cases, $VPAC_2$ receptors were expressed in 16/19 tested cases and CCK_2 receptors were found in 12/19 cases, whereas CCK_1 receptors were present in only 3/19 cases. The extraordinarily high density of all three receptors was remarkable, with densities above 2,000 dpm/mg tissue being observed for GRP receptors in 10/19 tumours, for

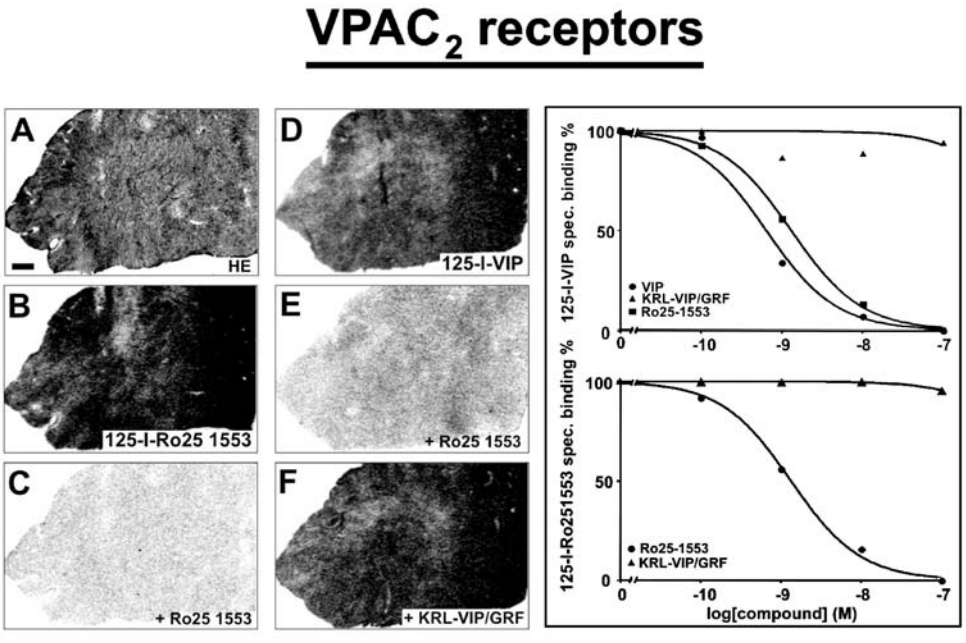

Fig. 2A–F. Characterisation of $VPAC_2$ receptors in GIST. Receptor autoradiography showing a tumour (**A** haematoxylin-eosin stained section, *bar*=1 mm) expressing $VPAC_2$, measured either with the $VPAC_2$-selective radioligand ^{125}I-Ro25-1553 [**B** total binding; **C** non-specific binding (in the presence of 20 n*M* unlabelled Ro25-1553)] or with the universal radioligand ^{125}I-VIP (**D** total binding) displaced by 20 n*M* of Ro25-1553 (**E**) but not by 20 n*M* of the $VPAC_1$-selective KRL-VIP/GRF (**F**). The *two graphs on the right* show complete displacement curves either with ^{125}I-VIP as universal ligand (*upper graph*) displaced with nanomolar concentrations of VIP (●) and Ro25-1553 (■) but not KRL-VIP/GRF (▲), or with ^{125}I- Ro25-1553 (*lower graph*) displaced by Ro25-1553 (●) but not by KRL-VIP/GRF (▲)

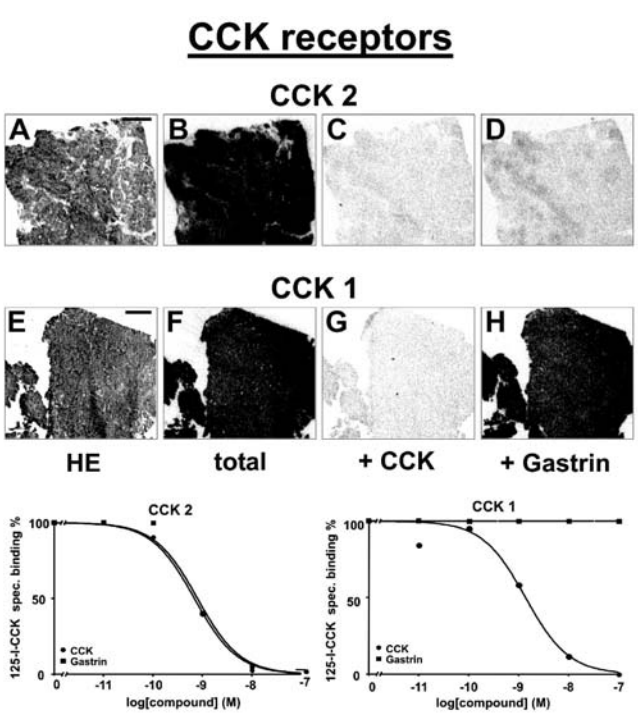

Fig. 3A–H. Characterisation of CCK receptors in GIST. *Upper part*: Receptor autoradiography of a CCK_2 receptor-expressing (**A–D**) and a CCK_1 receptor-expressing (**E–H**) GIST. **A, E** Haematoxylin-eosin stained sections. *Bars* =1 mm. **B, F** Autoradiograms showing total binding of ^{125}I-CCK. **C, G** Autoradiograms showing binding of ^{125}I-CCK in the presence of 50 n*M* CCK. Full displacement is seen in both cases. **D, H** Autoradiograms showing binding of ^{125}I-CCK in the presence of 50 n*M* gastrin. Displacement is seen in **D**, but not in **H**, indicating CCK_2 receptors in the upper case and CCK_1 in the lower case. *Bottom part*: Complete displacement curves in a CCK_2-expressing (*left*) and a CCK_1-expressing (*right*) GIST. In both cases, ^{125}I-CCK was displaced by nanomolar concentrations of CCK (●), whereas it was displaced by gastrin (■) only in the left case

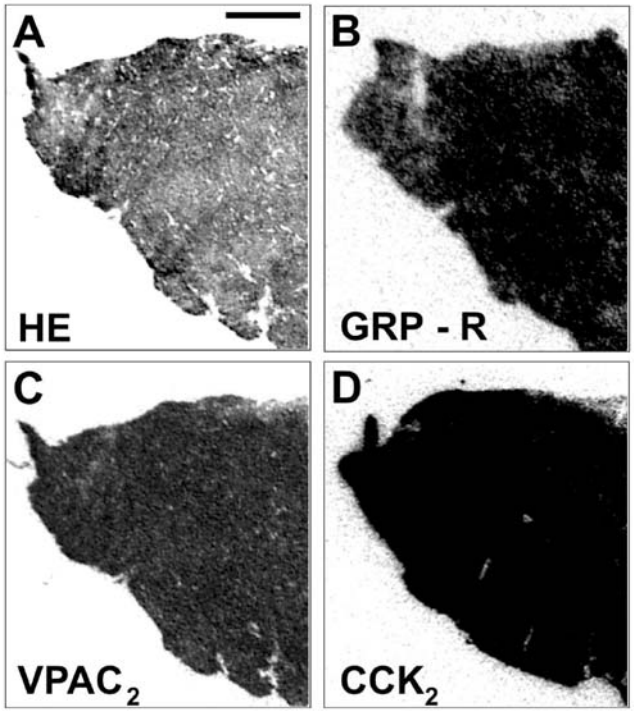

Fig. 4A–D. GIST (**A** haematoxylin-eosin stained section, *bar*= 1 mm) expressing concomitantly a high density of GRP receptors (**B** autoradiogram showing total binding of ^{125}I-[D-Tyr^6, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14)), $VPAC_2$ receptors (**C** autoradiogram showing total binding of ^{125}I-Ro25-1553) and CCK_2 (**D** autoradiogram showing total binding of ^{125}I-CCK). Full displacement was seen with the respective subtype-selective analogues (data not shown)

$VPAC_2$ receptors in 9/19 tumours and for CCK_2 receptors in 9/19 tumours. In 16/19 tested GIST (84%), at least one of these receptors was expressed with a very high density. In many cases, the measured densities of GRP, $VPAC_2$ and/or CCK_2 receptors reached levels higher than those usually found for the somatostatin receptors in neuroendocrine gastroenteropancreatic tumours [28]. Conversely, sst_2 receptors, NK_1 receptors, NMB (BB_1), BB_3 and NPY receptors were found only rarely in GIST, and, if present, usually in low to moderate density. Compared with $VPAC_2$, $VPAC_1$ was rarely expressed, and then only in low amounts. Furthermore, as reported previously for other tumour types, vessels expressing one or several peptide receptors, in particular NK_1 or/and NPY receptors, were frequently found in GIST. Figures 1, 2 and 3 show examples of tumours expressing some of the most frequently found peptide receptors in GIST, namely GRP, $VPAC_2$ and CCK receptors. Figure 1 shows an example of a tumour expressing GRP receptors (BB_2). Figure 2 is an example of a tumour expressing a high density of $VPAC_2$ receptors. Figure 3 illustrates two tumours having CCK receptors, one with CCK_2, the other with CCK_1 expression. In all examples, the pharmacological characteristics of the various receptors are also presented in competition experiments. Figure 4 shows a typical example of a GIST expressing concomitantly the three receptors, GRP, $VPAC_2$ and CCK_2.

No correlations were found between receptor status and any of the listed tumour characteristics, such as tumour size, mitotic index or presence of tumour necrosis (Tables 1, 2). There was no correlation between the receptor expression and the tumour localisation. However, it should be mentioned that peptide receptors could be identified in primary GIST in all locations, namely stomach, small intestine and mesentery. It is, moreover, important to note that all GIST metastases expressed concomitantly at least two of the peptide receptors in very high amounts. Finally, it should be stressed that the two patients with a terminal condition that had been treated prior to sampling (case 19 with Glivec, case 17 with chemo-embolisation) both retained a very high density of several peptide receptors (Table 2).

Discussion

We report for the first time that GIST express high levels of peptide receptors. These results may have an important and immediate clinical impact. It should indeed be possible to take advantage of the expression of these receptors to target GIST in vivo. Two different strategies should be considered: first, the development of in vivo receptor targeting of GIST for diagnostic purposes, namely for the precise localisation and the early detection of GIST recurrences and metastases, which still represent a difficult clinical problem, especially after anti-tyrosine kinase receptor therapy. Second, the development of peptide receptor radiotherapy, either as an alternative to Glivec or as an adjuvant treatment, for cases not responding or developing resistance to this drug. Peptide receptor targeting of GIST for diagnostic purposes could be successful in the relatively near future since the basic methods have already been developed and are available for two of the three peptide receptors most often expressed in GIST. There is indeed good evidence that GRP receptor-positive tumours can be localised in vivo with radiolabelled bombesin analogues. Van de Wiele et al. [17] were the first to report the visualisation of breast cancer; Scopinaro et al. [18] detected small prostate cancer metastases with this method. Moreover, CCK_2 receptor-positive medullary thyroid carcinomas (MTC) were successfully visualised with radiolabelled gastrin or CCK analogues [19, 20]. The fact that GIST express a density of CCK_2 receptors much higher than MTC, and a density of GRP receptors equal to or higher than prostate and breast cancers, is a strong argument for predicting successful visualisation of the smallest GIST recurrences and metastases. Finally, methods for peptide receptor radiotherapy have been developed that could be applied to GIST. The best evidence has been provided by the somatostatin receptor radiotherapy of neuroendocrine tumours using radiolabelled octreotide derivatives; studies from various centres agree on a 25% remission rate and a 60% stabilisation rate for somatostatin receptor-expressing neuroendocrine tumours using current protocols [11, 14, 15, 16]. Moreover, in a small series of MTC patients, CCK_2 receptor radiotherapy was also found to be successful [29]. Although there is as yet no in vivo evidence of successful $VPAC_2$ targeting of human cancers, specific $VPAC_2$ analogues are available [30] which should be developed as radioligands in order to take advantage of the very large number of $VPAC_2$ in GIST. Such $VPAC_2$ receptor scintigraphy may, compared with VIP receptor scintigraphy using ^{125}I-VIP, be characterised by a much lower background of normal organs, most frequently expressing $VPAC_1$ receptors [10]. Ultimately, if successful at a single level, one may try multi-receptor targeting [10, 28] as a potentially more powerful strategy, including CCK_2 receptor targeting in combination with GRP receptor targeting, and, once developed for clinical use, with $VPAC_2$ targeting.

With respect to the utilisation of the radiolabelled compounds for diagnosis and therapy, an appropriate assessment of the tumour to background ratio for the respective receptors should be helpful. Such an assessment is, however, difficult in the gastrointestinal tract, since the background consists, in this complex organ, of several different kinds of normal tissue (mucosa, muscles, nerves, immune cells) with a distinct peptide receptor expression that may also differ from one gut area to the other. Based on in vitro data, the following can be stated: $VPAC_2$ are primarily distributed in the gut smooth muscles and vessels [21], but not in the mucosa, which expresses $VPAC_1$ [25]. CCK_2 receptors are located in the smooth muscles and mucosa, very predominantly in the stomach [21, 31]. GRP receptors are located in the gut smooth muscles and nerves, but not in the mucosa [21]. Although the receptor density reaches significant levels in selected normal human tissues (e.g. CCK_2 in stomach), GIST tumours appear in general to express a higher density of the respective receptors. Furthermore, according to recent in vivo targeting studies in humans, the presence of peptide receptors in the normal gastrointestinal tract does not seem to affect significantly the scintigraphic evaluation, an exception being the CCK_2 receptors in the stomach: With ^{123}I-VIP scintigraphy (identifying $VPAC_1$ and $VPAC_2$), no clinically relevant uptake of the tracer was obtained in the normal intestinal mucosa [32], while using CCK2 receptor scintigraphy, strong uptake was detected in the stomach but not in other parts of the gastrointestinal tract [33]. GRP receptor scintigraphy with ^{99m}Tc-RP527 did not identify specific uptake in the intestines, although enterohepatic clearance of this tracer affected the interpretation of the scans at this level [17, 34].

The fact that the cells of origin of GIST, the Cajal cells [1], express several of these receptors in physiological conditions is a possible explanation for the expression of these receptors in GIST. It remains unclear, however, why the GRP receptors, the $VPAC_2$ receptors and the CCK_2 receptors are expressed in such a high incidence and amount, whereas other receptors, also expressed in Cajal cells, such as the sst_2 and NK_1 receptors, are only occasionally found in these tumours.

The very strong expression of three peptide receptors in GIST also has potential biological implications. First, GRP, $VPAC_2$ and CCK_2 receptors can be added to the number of known biological markers characterising this type of tumour. Second, knowing the strong growth-stimulating properties of GRP, VIP and CCK [35, 36], it is possible that all three peptides influence GIST growth through their respective receptors. Thus, in addition to activating mutations in the KIT receptor tyrosine kinase, and to the recently characterised activating mutations in the platelet-derived growth factor receptor [37], overexpression of peptide receptors may also have a significant pathogenetic role in the progression of GIST.

In conclusion, our in vitro study shows that most GIST express GRP, $VPAC_2$ and/or CCK_2 receptors. Al-

though it is clear that successful in vivo application of the present data will also depend on a variety of additional criteria [38, 39], the present receptor data predict that radiolabelled bombesin, vasoactive intestinal peptide and/or cholecystokinin analogues could be used as targeting agents to localise GIST in patients by scintigraphy. They also suggest that targeted radiotherapy with these radiolabelled peptides may offer an effective alternative to GIST treatment.

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