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HIGHLY INCREASED 125I-JR11 ANTAGONIST BINDING IN VITRO REVEALS NOVEL INDICATIONS FOR SST2 TARGETING IN HUMAN CANCERS

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Running title: New tumor targets with sst2 antagonist

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ABSTRACT

There is recent *in vitro* and *in vivo* evidence that somatostatin receptor sst_2 antagonists are better tools to target neuroendocrine tumors (NET) than sst_2 agonists. Indeed, antagonists bind to a greater number of sst_2 sites than agonists. Whether sst_2 antagonists could be used successfully to target non-NET tumors, expressing low sst_2 density, is unknown. Here, we compare quantitatively ^{125}I-JR11 sst_2 antagonist binding *in vitro* with that of the sst_2 agonist ^{125}I-Tyr^3-octreotide in large varieties of non-NET and NET. Methods: *In vitro* receptor autoradiography was performed with ^{125}I-JR11 and ^{125}I-Tyr^3-octreotide in cancers from prostate, breast, colon, kidney, thyroid, and lymphoid tissues, as well as NETs as reference. Results: In general, ^{125}I-JR11 binds to many more sst_2 sites than ^{125}I-Tyr^3-octreotide. In 13 breast cancers, 8 have low binding (mean density: 844±168 dpm/mg tissue) with the agonist while 12 have a high binding (mean density: 4447±1128 dpm/mg tissue) with the antagonist. All 12 renal cell cancers (RCC) show a low binding of sst_2 with the agonist (mean density: 348±49 dpm/mg tissue) while all cases have a very high sst_2 binding with the antagonist (mean density: 3777±582 dpm/mg tissue). 1/5 medullary thyroid cancers are positive with the agonist, while 5/5 are positive with the antagonist. In 15 Non-Hodgkin lymphomas (NHL), many more sst_2 sites are labelled with the antagonist than with the agonist. In 14 prostate cancers, none have sst_2 binding with the agonist and only 4 have a weak binding with the antagonist. None of 17 colon cancers show sst_2 sites with the agonist and only 3 cases are weakly positive with the antagonist. In the various tumor types, adjacent sst_2-expressing tissues such as vessels, lymphocytes, nerves, mucosa or stroma were more strongly labelled with the antagonist than with the agonist. The reference NET cases, incubated with a smaller amount of tracer, were also found to have many more sst_2 sites measured with

the antagonist. Conclusion: All RCC, a majority of breast cancers, NHL and medullary thyroid cancers represent novel indications for the *in vivo* radiopeptide targeting of sst_2 by sst_2 antagonists, comparable to NET radiotargeting with sst_2 agonists.

INTRODUCTION

Somatostatin receptors (sst) are highly over-expressed in gastroenteropancreatic and extra-gastrointestinal neuroendocrine tumors (NET). This represents the molecular basis for sst targeted diagnostic and therapeutic procedures in NET patients with somatostatin analogs (*1-3*). One main clinical application makes use of the inhibitory effects of somatostatin on neuroendocrine tumor cells, particularly on hormone secretion: Long-acting somatostatin analogs, such as octreotide or lanreotide, potently inhibit tumoral hormone secretion and improve related symptoms. The second targeting approach is based on the administration of radioactive somatostatin analogs for diagnostic or therapeutic purposes. Indeed, ^{111}In-octreotide scintigraphy (OctreoScan®) and other sst_2-targeted imaging with e.g. ^{68}Ga DOTA-TATE are gold standard methods for NET detection. Moreover, somatostatin receptor radionuclide therapy of NET with ^{90}Y- or ^{177}Lu-labeled somatostatin analogs is highly effective and increasingly applied (*4, 5*). Recently, results of the first prospective, randomized, controlled phase III study using radiolabeled somatostatin analogs for peptide receptor radiotherapy, were presented. Lutathera (^{177}Lu-DOTATATE) significantly improved progression free survival in comparison with cold octreotide therapy in advanced midgut NET (*6*).

While it could be shown *in vitro* that sst_2 antagonists do not trigger receptor internalization (*7*), we could show in animal models that radiolabeled sst_2 or sst_3 antagonists are not only as good in targeting *in vivo* the respective receptor, but even considerably better than the respective agonists (*8*). The findings that antagonists for G-protein coupled receptors recognize more binding sites compared to agonists is not new and discussed in the literature since years by several groups (*9, 10*). There are only

hypotheses in regard to the origin of this difference. The difference is explained by distinct receptor states, those coupling to the G-proteins and un-coupled forms. Antagonists bind to all receptor states whereas agonists bind only to the G-protein-coupled forms that are assumed to represent only a small proportion of the total receptor population (*9*). In cancer, GPCRs are frequently overexpressed and the superiority of antagonists was shown for the GRP receptor (*11*), the somatostatin receptors (sst2,sst3)[8] and the glucose-dependent insulinotropic polypeptide receptor (*12*).

A first pilot study in man using a first generation sst_2 antagonist confirmed the animal studies (*13*). The antagonists were better than agonist for NET imaging (*13*). A parallel *in vitro* study on tissue sections fully confirmed that the ^{177}Lu-DOTA-Bass antagonist labels more sst_2 binding sites than the ^{177}Lu-DOTA-TATE agonist (*14*). This was observed in NET but, interestingly, also in a few non-NET tumors with a low number of sst_2 (*14*). Presently, second generation sst_2 antagonists such as JR11, are in clinical development with promising results for diagnosis and targeted radionuclide therapy (*15*).

In the present study, we aimed at evaluating the *in vitro* binding properties of the second generation antagonist JR11, labeled with ^{125}I, and compared it with ^{125}I-Tyr^3-octreotide, the standard agonist radioligand (*16, 17*). ^{125}I labeling is the optimal choice for *in vitro* receptor autoradiography quantification (*17, 18*). Indeed, ^{125}I-radioligands have been used since decades for receptor autoradiography measurements and quantification of the data using radioactive reference standards has been routinely performed (*18-20*). The present study therefore allows a direct comparison of quantification data with previously published studies using ^{125}I-radioligands (*18-20*). This is at difference with the previous ^{177}Lu-DOTA-Bass study (*14*) where the data using

^{177}Lu-labeled ligands could not be directly compared to data with ^{125}I-labeled ligands. In the present study, a large number of tumors, including such tumors with high density sst_2 (gastroenteropancreatic NET, pheochromocytomas) and such known to express little or no sst_2 (renal cell cancer, breast cancer, prostate cancer, non-Hodgkin lymphoma (NHL), medullary thyroid cancer, colon cancer) were tested with both ligands. Non-tumoral tissues, adjacent to the tumors, were also evaluated.

MATERIALS AND METHODS

In a first part, we have designed cold iodinated JR-11 (iodo-JR11) (structure: DOTA-Cpa-D-Cys-Aph(L-HOro)-D-Aph(Cbm)-Lys-Thr-Cys-D-3-iodo-Tyr-NH2) and measured its binding affinity to sst_2. Iodo-JR11 was then tested in a displacement experiment using sst_2 transfected cells in an *in vitro* autoradiography setting with ^{125}I-Tyr^3-octreotide as radioligand, as reported previously (*21*). IC_{50} values were 1.89 +/- 1.1 nM (n=3) for iodo-JR11and 2.83 +/- 1.6 nM (n=3) for the reference peptide SS-28. The compound iodo-JR11 has therefore high affinity binding to sst_2 and is suitable as radioligand for the planned cancer experiments.

In a second part, we have used ^{125}I JR-11 (2'000 Ci/mmol, Anawa, Switzerland) as radioligand to evaluate the presence of sst_2 in human breast cancers, renal cell cancers, NHL, medullary thyroid cancers, prostate cancers, colon cancers, pheochromocytomas, paragangliomas, bronchial and ileal NET, and small cell lung cancers. Comparison was done with the agonist tracer ^{125}I-Tyr^3-octreotide (2'000 Ci/mmol, Anawa, Switzerland), known to have similar IC_{50} than the antagonist (*21*). Quantification was performed as previously reported (*21*). Specific binding was calculated as total binding minus nonspecific binding for each tumor case. Nonspecific binding was assessed in each

case in an adjacent section incubated with ^{125}I JR-11 or ^{125}I-Tyr^3-octreotide in presence of 100 nM of the unlabeled antagonist or agonist, respectively. The study conformed to the ethical guidelines of the Medical Faculty of the University of Berne. The institutional review board approved this retrospective study and the requirement to obtain informed consent was waived.

RESULTS

One group of tumors, shown previously with sst_2 agonist tracers to have low (<1'000 dpm/mg tissue) levels or a lack of sst_2, consisting of 13 samples of breast carcinomas, 12 samples of renal cell cancers, 15 samples of NHL, 5 samples of medullary thyroid cancers, 14 samples of prostate cancers, and 17 samples of colon cancers were used in receptor autoradiography experiments as reported previously (*22-29*). Successive sections were incubated in a buffer solution containing the standard amount of 30'000 cpm/100 µl ^{125}I-Tyr^3-octreotide or 30'000 cpm/100 µl ^{125}I-JR-11. Quantification was performed as previously reported (*21*). Tables 1, 2, and supplemental Table 1 show all the results.

A second group of tumors, shown previously with sst_2 agonist tracers to have a high level of sst_2, consisting of 5 pheochromocytomas, 2 paragangliomas, 4 ileal NET, 1 bronchial NET and 4 small cell lung cancers were also used in receptor autoradiography experiments; however, instead of 30'000 cpm/100 µl of tracer, only 10'000 cpm/100 µl of ^{125}I-Tyr^3-octreotide or ^{125}I-JR11 were given. All these cases had been tested previously with 30'000 cpm/100 µl ^{125}I-Tyr^3-octreotide and shown to express a high density (>2'000 dpm/mg tissue) of receptors in all cases. The rationale for the strategy to use 10'000 cpm/100 µl radioligand is that we assume, based on Cescato et al (*14*), that the

antagonist tracer binds to more sites than the agonist, and that therefore it is mandatory to use a lesser amount of tracer in order to prevent overexposure of the films in the antagonist part of the experiment. The results are summarized in Table 3.

Breast cancers: Table 1 shows that 8 of 13 breast cancer samples express somatostatin receptors using ^{125}I-Tyr^{3}-octreotide, in the majority of the cases in low density. The mean density of the receptor-positive cases is 844 +/- 168 dpm/mg tissue (mean±standard error of the mean). These data are comparable with previously reported results (*22*). With ^{125}I-JR11, however, somatostatin receptors can be detected in as many as 12 of 13 breast cancers, often in very high density. The mean density of the receptor-positive cases is 4447 +/- 1128 dpm/mg tissue. Figure 1 illustrates the results. Interestingly, although the antagonist labels more sites, the heterogeneity of labeling seen with the agonist is not abolished with the antagonist (Table 1), indicating that breast cancers are multiclonal tumors with areas with and without somatostatin receptors. Moreover, the tumoral and peritumoral vessels in the breast cancer samples are sometimes (4/9 cases) weakly labeled with ^{125}I-Tyr^{3}-octreotide. However, with ^{125}I-JR11, all 9 cases with identified vessels show a moderate to high labeling of these vessels. Therefore, these results are strongly indicative for a markedly higher binding of ^{125}I-JR11 versus ^{125}I-Tyr^{3}-octreotide.

Renal cell cancers: Table 1 also shows in a series of 12 renal cell cancers a very low level of somatostatin receptors with the agonist in all cases; however, a high density of somatostatin receptors can be identified in all cases with the antagonist. One example is shown in Figure 1. Mean density value with the agonist is 348±49 dpm/mg tissue (mean±standard error of the mean) and 3777±582 dpm/mg tissue with the antagonist,

representing a more than 10 times difference. The density values with the antagonist resemble very much the levels seen in NET tumors in standard studies with the agonist (*18-20*). As seen previously in breast cancers, a much higher number of binding sites is detected in peritumoral vessels with the antagonist than with the agonist.

Medullary thyroid cancers: Table 2 reveals that 4/5 medullary thyroid cancers are not labeled with the agonist, but that all show moderate to high labeling with the antagonist. Figure 1 shows a representative example.

Non-Hodgkin lymphoma: Similarly, Table 2 shows that in the 15 NHL the antagonist tracer binds to massively more sst_2 than the agonist. Whereas the values for the agonist are low or negative, the values measured with the antagonist can be compared to what is seen in NET with the agonist in standard studies (*18-20*). Figure 1 shows a representative example. Here again, tumoral vessels are strongly positive with the antagonist.

Prostate cancers: Supplemental Table 1 shows that the agonist ^{125}I-Tyr^3-octreotide does not detect any sst_2 in the 14 prostate cancer samples, confirming previously published results (*23*), while the antagonist ^{125}I-JR11 detects 5 positive cases, 4 of them having only a low sst_2 density. Therefore, the antagonist has a higher sensitivity to detect sst_2 in prostate cancer than the agonist. The higher sensitivity is also observed in the normal adjacent prostate tissues, such as stroma, vessels or nerves: indeed, while these tissues are occasionally labeled with ^{125}I-Tyr^3-octreotide, as reported previously as well (*23*), they are labeled to a much higher level in the experiments with ^{125}I-JR11. Figure 2 summarizes these results.

Colon cancers: Supplemental Table 1 also shows that colon cancers in general do not express significant quantities of sst_2 receptors, neither with the agonist nor with the antagonist tracer (Figure 1), except for 3 cases with borderline levels of receptors as measured with the antagonist radioligand; this density is probably not clinically relevant for tumor targeting. However, in approximately half of the agonist experiments, tumoral vessels, lymphatic follicles and mucosa are labeled as known from previous studies (*30*); in the antagonist experiments, not only are the vessels, lymphatic follicles and mucosa labeled in all tested samples, but the amount of labeling in these compartments is much higher than with the agonist.

NET: In the series of tumors (Table 3) representing classical indications for somatostatin receptor targeting *in vivo*, based on the high number of somatostatin receptors, namely pheochromocytomas, paragangliomas, ileal and bronchial NET and small cell lung cancers, we have compared agonist and antagonist tracers using a lower amount of tracer of 10'000 cpm/100 µl in order to prevent overexposure of the films using the antagonist tracer. Despite of the lower radioactivity application, the antagonist tracer binds with high levels while the agonist tracer binds in general with low levels, as seen in an example in Figure 1. The difference in binding between antagonist and agonist varies between 2.5 fold and 40 fold.

DISCUSSION

The comparison of the binding of the sst_2 agonist ^{125}I-Tyr^3-octreotide with the sst_2 antagonist ^{125}I-JR11 shows impressively that the antagonist labels many more sst_2 binding sites than the agonist, in tumor cells as well as in adjacent sst_2-expressing tissues. The breast cancer data confirm and expand from the receptor quantification

aspect a previous paper (*14*) showing that a ^{177}Lu-labeled sst_2 antagonist labels more sites than a ^{177}Lu-labeled sst_2 agonist. Based on the high number of ^{125}I-JR11 binding sites (>2'000 dpm/mg tissue) in more than half of the tested cases, we can conclude in the present study that breast cancers should be added to the list of tumor types that may be successfully targeted in vivo with sst_2 antagonist tracers. A similar conclusion can be drawn for RCC; the antagonist shows a more than 10 times mean increase in sst_2 binding as compared to the agonist. Here, 10/12 cases show a sst_2 density higher than 2'000 dpm/mg tissue. Such values predict a positive sst_2 imaging *in vivo* in patients (*18*). Therefore, renal cell cancers may be considered a serious indication for radiopeptide targeting with sst_2 antagonists. In medullary thyroid cancer and NHL, the antagonist tracer labels many more sites than the agonist, with 2/5 medullary thyroid cancer and 8/15 NHL having an sst_2 density over 2'000 dpm/mg tissue, respectively. The marked differences between agonist and antagonist binding may be explained on the basis of G-protein coupling with the receptor, as discussed above. G-protein coupled receptors are present in at least two affinity states for the radioligands, a small proportion in a G-protein coupled receptor conformation can be labeled by agonists and a large proportion in a G-protein independent (uncoupled) receptor conformation can be labeled by antagonists (*9*).

Of note, it should be reminded that in the past, there have been *in vivo* patient studies using sst_2 agonist that have documented that sst_2 targeting may be feasible under certain conditions in a number of non-neuroendocrine tumors and/or tumors expressing only occasionally sst_2 receptors (*25-29*). Such *in vivo* studies in patients included targeting of breast cancers (*22, 31*), renal cell cancers (*32, 33*), NHL (*34*), prostate

cancers (*24*), and medullary thyroid cancers (*35*). However, as opposed to neuroendocrine tumors, there has never been a systematic follow-up and extension of these studies, because it was clear that the predominantly low number of sst_2 detected by the agonist radioligands would prevent a routine application of sst_2 ligands in these indications.

Other types of cancer tested in the present study do not appear to have a significant number of agonist or antagonist binding sites. Indeed, the prostate cancers are unlikely to be an adequate target for JR11, not only because the tumor cells express rarely somatostatin receptors but also because adjacent tissue, in particular stroma, vessels and nerves, regularly express somatostatin receptors that may mimic a tumoral sst_2 expression. Similarly, colon cancers seem not to be appropriate tumor types for sst_2 radiotargeting, neither with sst_2 agonist nor antagonists.

For comparison, the tumors with an established high density of sst_2, namely the NET in Table 2, show in all cases a massive overexpression of sst_2 with the antagonist, despite the fact that in the present experimental setting the tumor sections were incubated with only one third of the standard radioligand dose. These data confirm what is seen *in vivo* in patients with gastroenteropancreatic NET using the sst_2 antagonist JR11, as reported by Wild et al. (*15*). Moreover, not only gastrointestinal NET, but also pheochromocytomas, paragangliomas and lung neuroendocrine tumors may be ideally targeted with sst_2 antagonists.

CONCLUSION

While the present *in vitro* data convincingly show that sst_2 antagonists of the second generation like JR11 are successful and superior to agonists in NET, it also strongly suggests that new indications for sst_2 targeting with these antagonists should be seriously considered, namely renal cell cancers, breast carcinomas, medullary thyroid cancer and NHL. These *in vitro* data provide the molecular basis to initiate a clinical trial in these indications.

DISCLOSURE

Jean Claude Reubi is a consultant to Ipsen and is inventor of several licensed patents. Helmut Mäcke and Jean Rivier are inventors of licensed patents.

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FIGURE LEGENDS

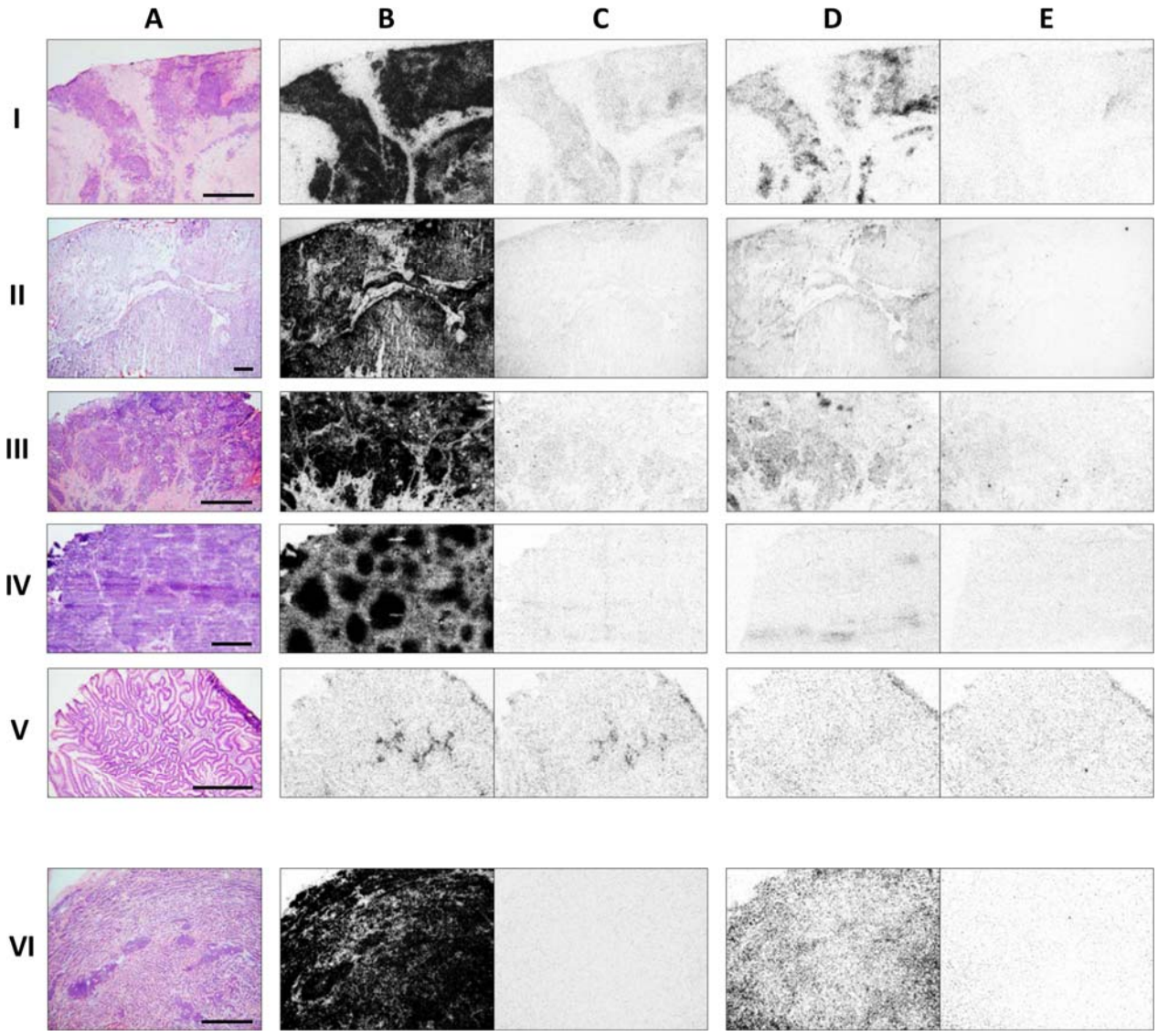

Figure 1

Comparative receptor autoradiography in various cancer types (A = HE staining) with ^{125}I-JR11 (B+C) and ^{125}I-Tyr^{3}-octreotide (D+E). B and D are the respective total binding, C and E the respective non-specific binding. Bars = 1 mm.

I: Breast cancer. II: Renal cell cancer. III: Medullary thyroid cancer. IV: Non-Hodgkin lymphoma. V: Colon cancer.

In cancers I-V, the sections were incubated with 30’000 cpm/100 µl of antagonist or agonist. Cancers I-IV show a much higher density of sst_2 with the antagonist. The colon cancer V is negative.

VI: Ileal NET, incubated with 10’000 cpm/100 µl of antagonist or agonist.

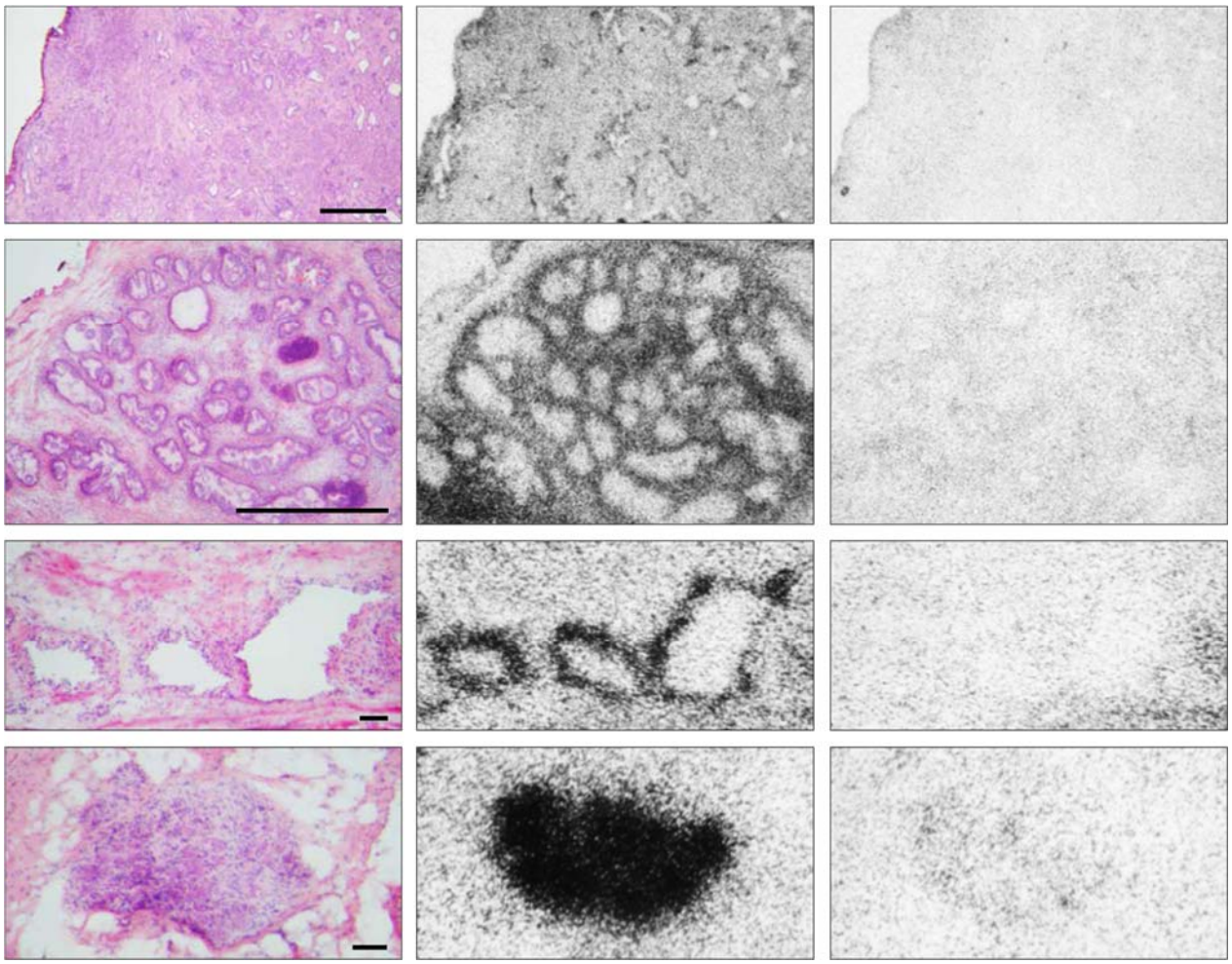

Figure 2

Receptor autoradiography with ^{125}I-JR11 showing the somatostatin receptors in a prostate cancer sample.

Left column: HE staining. Middle column: total binding of JR11. Right column: non-specific binding.

Upper row: weakly receptor-positive prostate cancer. Bar = 1 mm.

Second row: receptor-positive stroma. Bar = 1 mm.

Third row: receptor-positive vessels. Bar = 0.1 mm.

Lower row: receptor-positive nerve. Bar = 0.1 mm.

TABLES

Table 1: Comparison of ^{125}I-JR11 antagonist binding with ^{125}I-Tyr^3-OCT agonist binding in breast and renal cell cancers using 30'000 cpm/100µl radioligand.

Case No.	Tumor tissue		Surrounding vessels	
	^{125}I-JR-11 spec.bind; dpm/mg tissue	**^{125}I-Tyr^3-OCT spec.bind; dpm/mg tissue**	**^{125}I-JR-11 spec.bind; dpm/mg tissue**	**^{125}I-Tyr^3-OCT spec.bind; dpm/mg tissue**
Breast Ca				
No. 1	3648	394	4632	1465
No. 2	2179 het	417 het		
No. 3	283	0	1861	508
No. 4	9937	1535	4302	0
No. 5	1891 het	382 het	2497	0
No. 6	881	0	2062	0
No. 7	4226 het	508		
No. 8	420	0	2196	591
No. 9	1883 het	0		
No. 10	0	0	2236	0
No. 11	8788	958		
No. 12	9225	1349	3325	559
No. 13	>10'000	1207	3512	0
Renal cell Ca				
No. 1	3833	157		
No. 2	6083	388		
No. 3	4871	693	5471	1898
No. 4	8169	209		
No. 5	4932	656		
No. 6	1931	236	3908	1056
No. 7	2008	337	6451	1100
No. 8	4609	311		
No. 9	1631	165	2855	920
No. 10	2106	345	3723	1245
No. 11	2391	311	3659	1224
No. 12	2761	368	3914	1565

het; heterogeneous receptor distribution. Nonspecific binding values measured in 13 breast carcinomas were 613±73 dpm/mg tissue (mean±SEM) in the ^{125}I-JR11 experiments and 346±24 dpm/mg tissue in the ^{125}I-Tyr^3-OCT experiments. Nonspecific binding values in 12 renal cell carcinomas were 397±31 dpm/mg tissue (mean±SEM) in the ^{125}I-JR11 experiments and 270±14 dpm/mg tissue in the ^{125}I-Tyr^3-OCT experiments.

Table 2: Comparison of ^{125}I-JR11 antagonist binding with ^{125}I-Tyr^3-OCT agonist binding in medullary thyroid cancers and non-Hodgkin lymphomas using 30'000 cpm/100µl radioligand.

Case No.	Tumor tissue		Surrounding vessels	
	^{125}I-JR-11 spec.bind; dpm/mg tissue	**^{125}I-Tyr^3-OCT spec.bind; dpm/mg tissue**	**^{125}I-JR-11 spec.bind; dpm/mg tissue**	**^{125}I-Tyr^3-OCT spec.bind; dpm/mg tissue**
Medullary thyroid Ca				
No. 1	1763	0	3059	275
No. 2	1771	0		
No. 3	650	0		
No. 4	3963	499		
No. 5	2720	0	3437	643
Non-Hodgkin lymphoma				
No. 1	1956	184		
No. 2	6066 het	580 het	3626	853
No. 3	1815	0		
No. 4	2454	251	3387	913
No. 5	4589	697	4489	1593
No. 6	1976	0	3576	0
No. 7	2895	405	4501	652
No. 8	1308	0	3887	238
No. 9	1307	0		
No. 10	6208 het	446 het		
No. 11	1425	0		
No. 12	2070	186	5296	551
No. 13	495	0	4645	0
No. 14	5829	465	4030	1124
No. 15	4680	0	4546	0

het; heterogeneous receptor distribution. Nonspecific binding values in medullary thyroid carcinoma were 837±79 dpm/mg tissue (mean±SEM) in the ^{125}I-JR11 experiments and 613±54 dpm/mg tissue in the ^{125}I-Tyr^3-OCT experiments. Nonspecific binding values in non-Hodgkin lymphoma were 664±42 dpm/mg tissue (mean±SEM) in the ^{125}I-JR11 experiments and 407±26 dpm/mg tissue in the ^{125}I-Tyr^3-OCT experiments.

Table 3: Comparison of ^{125}I-JR11 antagonist with ^{125}I-Tyr^3-OCT agonist in selected tumors with a high density of sst_2 receptors using 10'000 cpm/100µl radioligand.

Case No.	Tumor tissue		Surrounding vessels	
	^{125}I-JR-11 spec.bind; dpm/mg tissue	^{125}I-Tyr^3-OCT spec.bind; dpm/mg tissue	^{125}I-JR-11 spec.bind; dpm/mg tissue	^{125}I-Tyr^3-OCT spec.bind; dpm/mg tissue
Pheo No. 1	>10'000	144		
Pheo No. 2	7893	335		
Pheo No. 3	8205	1545		
Pheo No. 4	4659	0		
Pheo No. 5	8502	207		
Paraganglioma No. 1	>10'000	810		
Paraganglioma No. 2	>10'000	472	1807	273
NET ileal No. 1	6124	597		
NET ileal No. 2	7756	830		
NET ileal No. 3	>10'000	3436		
NET ileal No. 4	>10'000	4167		
NET bronchial No. 5	4268	672		
SCLC No. 1	>10'000	1719		
SCLC No. 2	>10'000 het	3754 het	1959	0
SCLC No. 3	5517	708	1865	0
SCLC No. 4	5517	708	1865	0

het; heterogeneous receptor distribution. In all cases, ^{125}I-JR11 and ^{125}I-Tyr^3-OCT were given at a dosis of 10'000 cpm/100ml incubation solution.