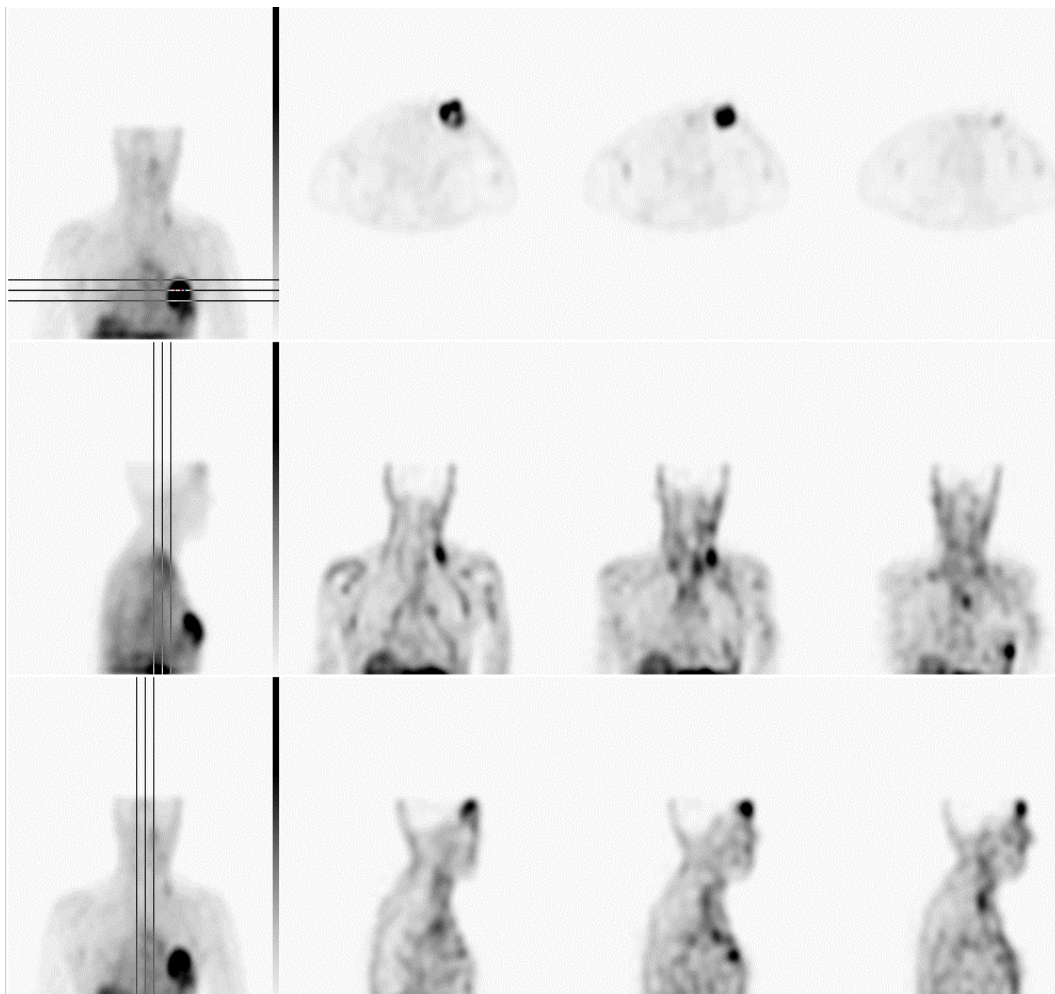Universiteit Gent
Faculteit Geneeskunde en Gezondheidswetenschappen
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NOVEL APPROACHES TO RECEPTOR IMAGING IN BREAST CARCINOMA : METHODOLOGICAL AND PRELIMINARY CLINICAL EVALUATIONS

**Proefschrift ter verkrijging van het
Doktoraat in de Medische Wetenschappen**

Door
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RECEPTOR IMAGING IN BREAST CARCINOMA : FUTURE PROSPECTS

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I. INTRODUCTION

Breast cancer is by far the most common cancer among women and after lung cancer the most lethal, accounting for 22% of all cancer deaths (1) . The vast majority of deaths are caused by the effects of metastasis, i.e. stage IV disease. The lifetime odds of getting breast cancer now stand at 1 in 8, double the risk of 1940 (2).

The most common clinical question in breast cancer concerns the risk of systemic recurrence after definitive primary therapy. This risk estimation is aimed at defining the need for systemic adjuvant therapy and patient prognosis. The classical basis of prognosis in breast cancer patients is the clinical and pathological Tumor Node Metastasis (TNM) staging, with the presence or absence of lymph node involvement being the most important prognostic indicator in primary breast cancer. Histologic grading, patient age with age under 35 being associated with poorer outcome and α-estrogen receptor (αER) and progesteron receptor (PR) assessment are not strong predictors of prognosis, but confer a modest effect on outcome, independent of tumour size and nodal status (3,4,5,6). Ongoing efforts to harness novel prognostic markers are aimed at fine tuning of prognosis in both node positive and node negative patients.

The second most frequently asked question is whether information can be obtained to predict which therapy might be particularly advantageous. In this regard, the value of intracellular αER and PR status for predicting response to hormonal treatment is well documented (7).

Given both the prognostic and particularly the predictive value of αER and PR in human breast cancer, several analogs of estrogens and progestins have been radiolabelled and evaluated in vivo for receptor binding affinity and selectivity. Few of these ligands, however, reached the clinical stage. These include halogenated substituents of estradiol and vinylestradiol (8,9,10). The clinical potential of these ligands has been recently reviewed (8). Overall, currently available data in humans do not substantiate a quantitative relationship between uptake of these estradiol radiolabelled agents and the content and binding characteristics of αER

Recent major advances made possible by the development of new investigational techniques have broadened the interest from the intracellular αER and PR receptor to cell surface receptors and their cognate ligands that are upregulated in breast carcinoma and participate in the aberrant growth of breast cancer. These cell surface receptors are subdivided in 3 superfamilies : (1) proteins formed of a core structure of seven transmembrane α-helical sequences with intracellular coupling to trimeric G-proteins, the so called G-protein coupled receptors (GPCR's) ; (2) large glycoproteins which generally possess a single transmembrane sequence and tyrosine kinase activity grouped into 4 families (see figure 2) on the basis of sequence homology of their kinase domains, their structure and the structural similarity of their ligands, the so called Tyrosine Kinase Receptors (TKR's); and (3) molecules which span the cell membrane once but do not have kinase activity, the non-tyrosine kinase receptors (NTKR's). The final common pathway for most of these receptor systems is activation of a kinase cascade culminating in activation of extracellular-signal-regulated kinase (ERK). This serine/threonine kinase translocates into the cell nucleus where it propagates the mitogenic signal by way of phosphorylating and activating appropriate transcription factors to induce the expression of genes necessary for initiating the cell division cycle (see figure 1) (11,12,13,14). The increased knowledge on the biological properties and the relationship of these potential oncogenic receptor systems to the clinical behaviour of breast cancer has led to the development of novel therapeutic strategies and could provide an impetus for the development of novel radioligands.

II. WHY NUCLEAR MEDICINE TO ASSESS CELL SURFACE RECEPTOR EXPRESSION WHEN OTHER METHODOLOGIES ARE ALREADY AVAILABLE?

Different methodologies, respectively northern blotting, ligand binding assays (LBA's), immunohistochemistry (IHC), reverse trancript polymerase chain reaction (RT-PCR) and in situ hybridisation (ISH), each with a great variation in cut-off values have been used to assess cell surface receptor status leading to conflicting data (vide infra) e.g. prognostic value of HER-2/neu. As the size of the average tumour continues to decrease due to a more performant screening, blotting and ligand binding assays requiring bulk tumour homogenates are no longer possible in most patients. Thus IHC, ISH and RT-PCR will become more and more important. These techniques suffer from several drawbacks (15). Semiquantitative analysis of immunohistochemically stained tissue sections is operator dependent, requiring both judgement and experience of the observer, and resulting in a suboptimal reproducibility. Also, most antibodies recognise more than one molecule or epitopes. ISH is labour intensive and requires high sensitivity of the probes used. RT-PCR may lack specificity due to false priming. Most importantly, none of these techniques estimates receptor functionality.

In contrast with these techniques and their shortcomings nuclear medicine offers several potential advantages. These include the in vivo whole body assessment capability for visualising "functional" receptor expression, the fact that radioligands are not subject to animal variation as is the case in antibody production, the absence of errors of sampling and tissue manipulation, as well as preparation, and the ability to address the intrinsic heterogeneity of receptor expression. However, to demonstrate these potential advantages, radioligands with very high tumour to background ratio's will be needed to counterbalance the inherently low resolution and related partial volume effect of our gamma camera systems. As agonist- GPCR or - TKR complexes are internalised with the ligand being trapped in lysosomes, the emphasis should be on agonist radioligands for this entrapment will prolong the residence time of the ligands, resulting in higher tumour to background ratio's than can theoretically be obtained with antagonists (16,17). Consequently, radiolabelled peptides, the naturally occurring agonists of GPCR's, TKR's and NTKR's are more likely to yield high tumour to background ratio's when compared to radiolabelled murine monoclonal antibodies which are not internalised .

III CELL SURFACE RECEPTORS UPREGULATED IN HUMAN BREAST CARCINOMA

STAGING AND FOLLOW-UP

GPCR's upregulated in human breast cancer specimens when compared to the surrounding normal breast tissue include the gastrin releasing peptide receptor (GRP-R), vaso-active intestinal peptide receptor (VIP-R) and somatostatin receptor (SS-R). Reported incidences of GRP-R, SS-R, specifically subtype 2, and VIP-R are 71%, 36-80% and 100 % respectively (18-23). Ligand binding to these receptors activates a G-protein, which in turn activates or inhibits an enzyme that generates a specific second messenger. Depending on the subtype, GPCR's stimulate mitogen activated protein kinases (MAPK's) by increasing phosphatidylinositol turnover leading to activation of proteinkinase C and Raf or via $G_{\beta\gamma}$ subunits, depending on their ability to stimulate phospholipase C resulting in activation of Ras (see figure 1.) (24).

Upregulated TKR's in breast cancer are shown in figure 2 (indicated in bold). The highest reported incidences are those of PDGFb-R (platelet derived growth factor-b receptor, 86-100%), VEGF–R (vasoendothelilal growth factor receptor, KDR subtype, 100%), IR (insulin receptor, > 80 %) and IGF1-R (insulin-like growth factor-type 1 receptor, 50-90%) (25-35). Significantly lower values were reported for the c-erb-B family, respectively 20-58 %, 17-30 %, 19-21 % for subtype 1 (EGF-R or epidermal growth factor receptor), 2 (HER-2 neu) and 3, and for the FGF-R (fibroblast growth factor receptor) subfamily, 32 % , 22 % and 4 % for subtype 4,1 and 2 (36-50). Unlike GPCR's, which interact directly with an associated protein, TKR's are linked indirectly to Ras via two proteins, growth receptor binding protein 2 (Grb2) and Son of sevenless (Sos) (see figure 1) and, with the exception of the type II subfamily, activated by ligand induced dimerization.

NTKR's upregulated in breast cancer include amongst others the leukemia inhibitory factor receptor (LIF-R) and the interleukin-2, 3, 5, 6 and 11 receptors (IL-2,3,5,6,11 R) (51-57). Reported incidences for the interleukin family vary from 53-100 %, the highest incidence being reported for the beta subunit of the IL2-R (100%)(53). For LIF-R this value is 80 % (51). Contrary to CPCR's and TKR's, NTKR's use multiple signalling pathways, the best known of which is the Jak-STAT pathway, a very rapid cytosol-to-nuclear signaling pathway.

Thus, given their high reported incidence of expression in human breast cancer, radioligands guided against PDGFb-R, VEGF-R , VIP-R and IL2-R (beta subunit) seem to be of particular interest for staging. However, several facts need consideration. First, reported data have almost exclusively been obtained on primary tumour tissue and no extensive data on variability of receptor

expression between different nodal or metastatic lesions for these receptors are available. Second, specifically for VIP-R, the potential for overestimation of lymph node involvement due to high physiological VIP-R expression in lymph nodes, in the CD3 zone around lymphoid follicles, was stressed by Reubi (23,57). Finally, whether or not the degree of amplification of these receptors holds steady throughout the course of disease and is similarly amplified in subsequent metastases as has been suggested for HER-2neu and EGF-R, a prerequisit for follow-up, needs to be resolved. Knowledge on these issues can only be obtained non-invasively by means of receptor radioligand scintigraphy .

PROGNOSIS ASSESSMENT

In terms of prognosis, the most thoroughly investigated cell surface receptors in breast carcinoma are HER-2/neu(c-erb-2) and EGFR and to a lesser extent IGF-1 R and IR.

Using gene amplification, evidence for a role for HER-2/neu as a predictor of poor disease free survival and overall survival in node positive patients was first provided by Slamon et al. in 1987 (58). Attempts to replicate these results however have had mixed success, with some studies observing that HER2-neu gene amplification was an independent predictor (59,60) and others failing to find any useful information (61-65). Using IHC, similar results were obtained with only one out of more than ten studies in node negative patients showing that HER-2/neu overexpression was an independent marker of poor prognosis (66-76). The role of EGFR and other cell surface receptors such as IR and IGF1-R, as prognostic factors in breast carcinoma is less well studied and even more controversial. Particularly for EGFR, multiple techniques have been used with great variation in the cut-off points used to define high and low risk subsets. In most, but not all, of these studies EGFR failed to be an independent predictor of disease free survival or overall survival in node positive breast carcinoma patients when mutivariate analysis was done (77). Of interest, a number of these papers found EGFR to have prognostic value in node negative breast cancer (78,79). Finally, a number of studies, using a variety of methodologies, suggest that IR and IGF-I R are independent favorable prognostic factors strongly related to disease free time in node negative breast cancer (80).

Thus, in general, it seems that untill additional validation studies are done with a well-standardized technique for measuring these receptors, the use of cell surface receptors in defining prognosis remains controversial and not-usefull in clinical routine. In this regard, the availability of a technique able to assess "functional" receptor status is a more than exciting prospect. On theoretical grounds such a technique is likely to be more adequate. Importantly, estimates of receptor expression by means of nuclear medicine should be expressed as a continuous and reproducible variable as much prognostic information is lost or poorly presented by simple dichotomy or point scoring systems. This will require a rigid, uniform and standardized acquisition and processing protocol. Large numbers of patients in well defined disease stages, uniformly treated should be included in these studies to ensure that the results will be statisitically significant. It will then be possible to perform subset analysis and arrive at meaningfull conclusions. With increasing economic pressures and regulation, results obtained will need to be included in large, prospective intergroup studies comparing the accuracy of all available methodologies as is e.g. currently the case for the different immunohistochemic methods described for HER-2 neu expression. This will not be an easy task given the fact that nuclear medicine has no antecedents in this field and the use of radioactivity is to our disadvantage.

PREDICTION OF RESPONSE TO THERAPY

The need for prediction of response to a certain therapy in patients suffering from breast carcinoma is twofold. First, the classical assessment of response, based on volumetric changes, as defined by the Union International Contre le Cancer (UICC) response criteria, require an observation period of 2-6 months. Thus, with the exception of response to hormonal treatment which can be predicted with an accuracy of 60-70 % based on αER and PR status, non-responders are deprived of a potentially beneficial alternative treatment option for several months. Second, with an increasing number of patients receiving adjuvant therapy, the availability of a test that can predict successfull adjuvant therapy is becoming increasingly important as this treatment aims to cure patients.

Recent direct and indirect evidence suggests that assessment of cell surface receptor status might offer at least a "partial" solution to both problems.

A. Response prediction to conventional treatment modalities

Adjuvant chemotherapy : The relationship between HER-2/neu expression and success of adjuvant doxorubicine containing chemotherapy was initially observed in several retrospective studies. These and more recent studies differ in type of chemo-hormonal agents used for adjuvant treatment for breast cancer, in techniques and scoring systems used for HER-2/neu overexpression and in evaluation and interpretation of data (81). Examples of these subtleties and discrepancies in interpretation and evaluation are the two large, recently published reports from the Cancer and Leukemia Group B (CALBG) 8541/8869 and the

National Surgical Adjuvant Breast and Bowel project (NSABP) B11. CALBG 8869 evaluated the relationship between HER-2/neu expression and dose response to doxorubicine (30 mg/m2 vs. 40 mg/m2 vs. 60 mg/m2 per cycle) in a total of 992 patients (82). The effect of dose intensity of doxorubicine on survival and disease-free survival was evident only in the HER-2/neu-positive group. Unlike the CALBG study, the NSABP-11 study provided a comparison of a treatment regimen plus or minus doxorubicine and, therefore, a direct test of an interaction between doxorubicin and HER-2/neu expression (83). In this trial, HER-2/neu overexpression was related to improvement in outcome to the same dose of doxorubicin 30 mg/m2 at the lower end of the dose response curve in CALBG 8541. The interaction between doxorubicin treatment and HER-2/neu overexpression was statistically significant for disease free survival and distant disease free survival. Thus it appears that response to doxorubicin-based adjuvant therapy depends on HER-2/neu status. However, a consenus on the most reliable, most reproducible and most predicitive method to determine HER-2/neu status is still mandatory.

Neoadjuvant chemotherapy : Archer et al. evaluated the relationship between HER-2/neu expression and the ability of anthracycline-based neoadjuvant chemotherapy to induce apoptosis of tumour cells (84). The assessment of apoptosis was made through an apoptosis index defined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL). Their results demonstrated that this apoptotic index increased significantly less in nine patients with HER-2/neu overexpressing tumours than in 230 patients with HER-2 negative tumours (34 % versus 245 % respectively).

Adjuvant endocrine treatment : Data regarding HER-2/neu expression and response to adjuvant tamoxifen are conflicting. Whereas the Naples GUN trial demonstrated that HER-2/neu expression implies a diminished response to adjuvant tamoxifen (85), the CALBG 8541and the NSABP B-11 trials did not demonstrate this interaction. These discordant findings likely relate to the use of different antibodies and different scoring systems for HER-2/neu expression assessment and suggest the need for reliable and reproducible methodologies to determine HER-2/neu expression. Ongoing intergroup studies by the North Central Cancer Treatment Group (NCCTG) and the NSABP trialists are now including prospective analysis of HER-2/neu expression by different methodologies while analyzing the role of trastuzumab (vide infra) in an adjuvant setting.

Endocrine treatment in metastatic disease : In a series by Neskovic et al. in 106 newly diagnosed patients, a high EGF-R status was found to be highly predictive for non-response to endocrine treatment in metastatic breast cancer patients (86).

B. Response prediction to novel treatment modalities

Currently designed cytostatic anti-cancer strategies interfering with the autocrine/paracrine cell surface receptor/hormone loops, include blocking monoclonal antibodies, peptide hormone antagonists, peptide/toxin fusion proteins and inhibitors of signal transduction.

Blocking monoclonal antibodies: Trastuzumab or muMAb 4D5 (Herceptine [R]), a murine monoclonal antibody against the extracelullar part of HER-2/neu given concomitantly with first line chemotherapy for metastatic breast cancer improves time to progression (7.6 vs. 4.6 months) and overall response (48% versus 32 %) when compared to chemotherapy alone in a phase III multinational study evaluating 469 patients (87). The one year survival data increased significantly with the addition of Herceptine (68 % vs. 79 %, p < 0.01). Patient eligibility in this study was determined by testing tumour specimens for overexpression of HER-2 neu by means of IHC using a 4 point scoring system (0-3+). Only patients with tumour score 2+ and 3+, the currently FDA approved patient group for Herceptine treatment, were included. Retrospective analysis suggested that the beneficial effect of Herceptine therapy was inferior in patients with a 2+ level of HER-2 protein expression when compared to patients with a 3+ level. Other studies suggest that patients with untreated or chemotherapy refractory metastatic breast cancer may also benefit from monotherapy with Herceptine (88,89). The major side effect of Herceptine is cardiotoxicity which becomes particularly relevant when combined with anthracycline (90). Specific guidelines for monitoring cardiac function in these patients are yet to be developped.

Other potential blocking antibodies are 2A11 and C225 which respectively recognise GRP and EGFR blocking their biological activity in both in vitro and in human in vivo studies (91,92). Yang et al. demonstrated that the assessment of GRP-R status using IHC allowed prediction of growth inhibition of several breast carcinoma cell lines to 2A11 treatment (93). Several other potential blocking antibodies against e.g. PDGF and VEGF have been descried and are currently under evaluation in tumour cell lines and xenograft models (94,95).

Peptide hormone antagonists : Currently, several GPCR antagonists are being tested in phase I clinical trials e.g. the (Arg6,D-Trp,9, MetPhe8) substance P analogue which may prove usefull for treating breast carcinoma patients. Of interest, several papers demonstrate downregulation of EGF-R following effective administration of GRP-R antagonists in animal tumour models suggesting

the tumour promoting effect of GRP involves transactivation and upregulation of EGF-R (96).

Peptide hormone/toxin or chemotherapy fusion proteins : Another possibility is to utilise peptide hormones to vehiculate toxic compounds to tumour cells. As such recombinant diphteria toxin (DAB389)/EGF, IL-2 etc…hybrids, receptor or SP fusion proteins and hybrid doxorubicin /bombesin(GRP) hybrids have been constructed to selectively kill tumour cells expressing the corresponding receptor (97,98). The possible value of these agents in therapy will not only depend on their receptor specific targeting potential, but also on the toxicity on normal cells expressing these receptors. Thus selectivity of these agents can be a problem.

Inhibitors of signal transduction : Finally, anti-growth factor therapy can be accomplished by inhibiting molecules, involved in growth factor receptor signal transduction. Some of these molecules are close to or have already entered clinical trials, respectively : .(1) molecules such as tyrphostins, quinazolines and 2-substituted aminopyrido(2,3-d) pirimidones which can selectively inhibit tyrosine kinases including EGFR and PDGFR and TKR inhibitors against VEGFR-2 (Flk-1/KDR) and basic FGFR acting as novel antiangiogenic anticancer agents, (2) Peptidyl phosphotyrosine analogs designed to bind at the Grb2 SH2 site inhibiting Grb2 binding to activated TKR's, (3) farnesyl transferase inhibitors, blocking anchorage of Ras to the plasma membrane thereby prohibiting its activation and (4) modulators of proteinkinase C (PKC) which is particularly upregulated in node-negative breast carcinoma patients (99-103). Growth inhibition in vitro and in vivo by these agents is associated with downregulation of the targeted receptor on its cell surface.

To maximize the benefit from new treatment paradigms, objective markers of therapeutic success are mandatory. From the data presented, it may be deduced that characterization of cell surface receptor systems in breast carcinoma will become increasingly important for therapy response prediction. With this regard, based on available data, targeted radioligands against HER-2/neu and EGFR are of major interest. When compared to IHC , RT-PCR and ISH, the potential for whole body assessment will be a major advantage as clinicians prefer information on all lesions over information on only one lesion.

Whether or not therapy response prediction will be feasible by means of one single scanning examination remains to be proven. In general, high ligand uptake, indirectly reflecting receptor functionality and number may allow separation of responders from non-responders. Imaging prior to treatment should be sequential as

an increase of tumour to background ratio's from early to late images reflects ligand internalisation and thus receptor functionality. However, in some tumours, levels of receptor expression are not proportional to the level of signaling. Low receptor levels with high signaling may occur as a result of breakdown of normal cellular asymmetry and spatial seggregation of the receptor and its ligand. For instance, EGFR's are predominantly located on the basal part of the cell membrane of epithelial cells protecting them from overstimulation by high concentrations of circulating EGFR binding ligands. As such, receptor redistribution with a minor increase in apicocellular receptor number may result in a major upregulation of signaling loops. In this group of tumours, more accurate information on response to treatment could be obtained by repeating the scintigraphic examination shortly after treatment as effective treatment would result in downregulation of the functional receptor probably from as early as one week post-treatment. Efficiency of GPCR targeted treatment could also be visualised by means of sequential EGFR or other TKR visualisation before and after treatment, given their transactivation of these receptor pathways.

IV. CONCLUSION :

Nuclear medicine has the potential to significantly alter treatment planning and monitoring by offering therapy response prediction or early response assessment in patients suffering from breast carcinoma as well as prognostic information, by means of visualising cell surface receptors. For this purpose radioligands, preferably peptides, with high tumour to background ratio's and rigid, uniform standardized protocols will be needed. The main cell surface receptors of interest are the TKR's , with a specific role for EGF-R and HER2-neu, followed by the GPCR's. To guarantee their place in clinical practice these ligands will need to be evaluated in large muticenter studies requiring collaboration from various nuclear medicine centers and the respective clinicians involved in the management of patients suffering from breast carcinoma.

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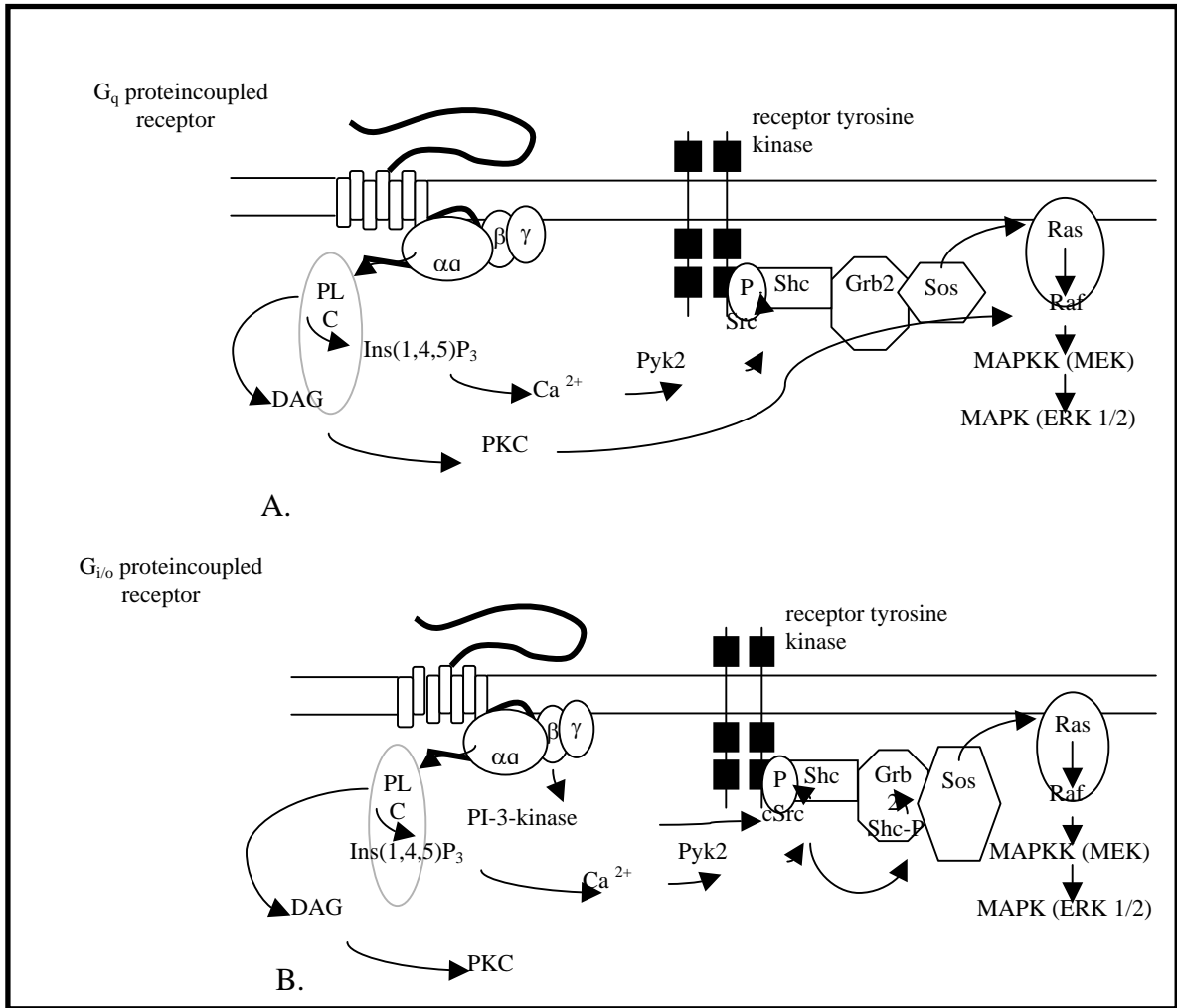
Figure 1 :

Ligand binding to the tyrosine kinase receptor generally results in receptor dimerization and autophosphorylation of cytosolic tyrosine residues which serve as docking site for the src homology 2 (SH2) domain in the growth receptor binding protein 2 (Grb2). The two SH3 domains in Grb2 then bind Son of Sevenless (Sos), a guanine nucleotide exchange factor, thereby bringing Sos clouse to membrane bound Ras. Ras is an intracellular switch protein that cycles between an inactive GDP-bound form and an active GTP-bound form. Binding of Sos to inactive Ras causes a large conformational change that permits release of GDP and binding of GTP. Thus activated Ras binds to the N-terminal domain of Raf, a serine threonine kinase which subsequently binds to and phosphorylates MEK, a dual-specificity protein kinase that phosphorylates both tyrosine and serine residues.

MEK than phosphorylates and activates MAP kinase (MAPK) which subsequently phosphorylates many different proteins, including nuclear transcription factors, that mediate cellular responses.

Ligand specfic binding to a G-protein coupled receptor leads to dissociation of GDP, which is replaced by GTP, bound to the α-subunit of the heterotrimeric G-protein. Subsequently G_{α}.GTP dissociates from $G_{\beta\gamma}$. Depending on the subtype, G-protein coupled receptors stimulate phospholipase C to release inositol (1,4,5)triphosphate(1,4,5)P3 and diacylglycerol (DG), which cause the release of intracellular Ca2+ and activate protini kinase C, either via G_{α}.GTP or via $G_{\beta\gamma}$ subunits. Through Pyk2 and Src, G-protein coupled receptors are linked to the MAPK pathway.
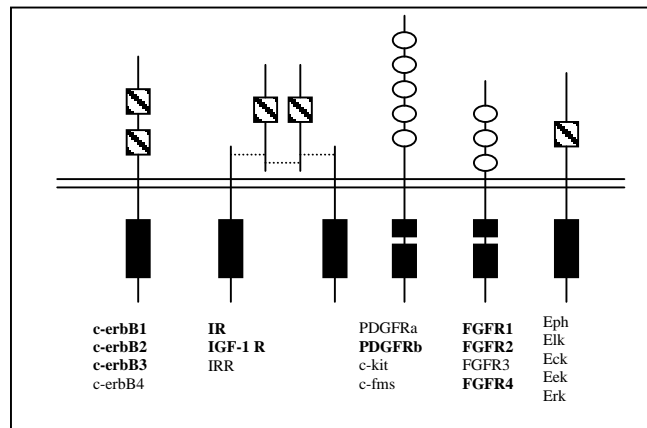
Figure 2.

Figure 2 shows the different tyrosine kinase receptor (TKR) families. Upregulated TKR's in breast cancer are indicated in bold. Abbreviations of relevance are explained in the text on staging and follow-up.

THESIS OUTLINE

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As early as 1936 Lacassagne proposed that if breast cancer was caused by a special hereditary sensitivity to estrogen, then the development of an estrogen antagonist could prevent the disease. Unfortunately, estrogen antagonists did not exist at that time and there was no therapeutic target. It was only in 1962 that the target was found by Jensen and Jacobson, who described the specific binding and retention of radiolabeled estradiol in estrogen target tissues. Jensen subsequently proposed the α estrogen receptor (αER) as the mechanism to modulate estrogen activity, leading to the development of numerous reversible and non-toxic endocrine deprivation treatment modalities of breast cancer. Even now, novel drugs targeted at blocking or reducing αER stimulation are about to enter the clinical stage including pure antiestrogens, potent and specific aromatase inhibitors and long acting preparations of LHRH analogues. Given the well documented value of intracellular αER status in human breast cancer for predicting response to these hormonal treatment modalities, several analogs of estrogens have been radiolabelled and evaluated in vivo for receptor binding affinity and selectivity. Few of these ligands however reached the clinical stage Unfortunately, available data on these halogenated substituents of estradiol and vynilestradiol in patients suffering from breast cancer fail to substantiate a relationship between uptake of these ligands and the content and binding characteristics of tumoural αER status as well as with outcome to hormonal treatment. As the mainstay of adjuvant- as well as palliative hormonal treatment for breast cancer is the anti-estrogen tamoxifen, assessment of radiolabelled tamoxifen uptake by means of nuclear medicine imaging might predict more accurately outcome to conventional hormonal treatment.

Recent major advances made possible by the development of new investigational techniques have broadened the interest from the intracellular αER receptor to cell surface receptors and their cognate ligand that are upregulated in breast carcinoma and participate in the aberrant growth of breast cancer. The increasing amount of knowledge on the role of these cell surface receptors in proliferative signal transduction has led to the development of novel cytostatic anti-cancer strategies interfering with these autocrine/paracrine cell surface receptor/hormone loops. One of the many studied ligand/cell surface receptor systems in breast carcinoma is the gastrin releasing peptide (GRP)/ gastrin releasing peptide receptor (GRP/GRP-R) pathway which may effectively be blocked by the murine monoclonal antibody 2A11.

This thesis describes the basic development of and the first methodological and clinical studies with 123Iodine–dimethyl-*N,N*-tamoxifen (ITX) and 99mTc RP527, radioligands for single photon emission tomographic imaging (SPECT) of human breast cancer, respectively targeted against the αER and GRP-R.

In part I the available data on radiolabelled estradiol derivatives to predict response to hormonal treatment in breast cancer patients are reviewed and studies with ITX described, considering both technical and clinical aspects.

In part II the rationale for GRP-R imaging in human carcinoma, including breast carcinoma, is reviewed and studies with 99mTc RP527 described, also considering technical and clinical aspects (phase I/II trials).

PART I : ESTROGEN RECEPTOR IMAGING IN BREAST CARCINOMA
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RADIOLABELLED ESTRADIOL DERIVATIVES TO PREDICT RESPONSE TO HORMONAL TREATMENT IN BREAST CANCER : A REVIEW

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ABSTRACT

Several radiolabelled steroidal and non-steroidal estradiol derivatives of which the tumoral uptake is believed to relate quantitatively to the content and binding characteristics of the αER receptor in the target tissue have been synthesized and their imaging potential and clinical usefulness evaluated in vivo in humans. Due to the use of different methodologies and cut-off values for measuring αER positivity, the use of both quantitative PET and semiquantitative SPECT, and the difference in patient populations studied, direct comparison of these data is not possible. Individual data however fail to substantiate a direct relationship between these radiolabelled estradiol derivatives and αER status in keeping with recent pathophysiological findings demonstrating 1) estradiol sequestration and retention through other than αER mediated, either membrane or non-membrane related, mechanisms and 2) an inverse relationship between estradiol uptake and local biosynthesis through aromatisation and interconversion in αER positive tumours. Additionally, given the discovery of very high affinity αER-like binding sites (Kd, dissociation constant, < 0.1 nM), and the potential for underestimation of αER Kd when using ligand binding assays, at least part of radiolabelled estradiol derivative uptake reflects tumoral perfusion rather than the ligand-receptor binding process.

However, the reduction in cellular uptake, membrane sequestration and local biosynthesis of estradiol following tamoxifen treatment in αER responsive tumors should allow early therapy prediction through rapid sequential radiolabelled estradiol scintigraphy with higher accuracy than conventional αER estimations as supported by recent data.

I. INTRODUCTION

Breast cancer is the most common malignant tumour among women comprising an estimated 24 % of all cancer cases, corresponding to an estimated 184,300 new cases per year in the European Community (1). Because of its propensity to metastasise, even before the disease can be detected clinically or by screening mammography, breast cancer remains the most lethal cancer for women, accounting for 18 % (58,000/ year) of all cancer deaths (2).

Based on three main lines of evidence : (a) the ability of estrogens to generate mammary tumours in rodents, (b) epidemiologically derived risk factors such as the protective effect of ovariectomy and increased risk of breast cancer in young women given diethylstilbestrol to prevent abortion and (c) the mitogenic effect of estrogens on established breast cancer cell lines and efficacy of antiestrogens in treating established breast cancer, estrogens are believed to be the major adverse factor in human breast cancer (3,4,5,6,7). Consequently, numerous modalities of endocrine therapy, primarily aimed at estrogen deprivation with comparable spectra of activity, either ablative, additive or antagonistic, have been developed (8,9,10). The main mechanism of action of these cytostatic agents which are now largely used for adjuvant or palliative purposes, either alone or in combination with chemotherapy, is believed to be a block of division of estrogen dependent tumour cells with arrest in the G0 or G1 phase of the cycle (11,12). Of these agents, the

principal and most frequently used is the anti-estrogen tamoxifen.

Tamoxifen is used both in an adjuvant setting as well as for treatment of metastasized breast carcinoma. Whereas older studies such as the NATO- and Scottisch trial favored the systematic use of tamoxifen treatment in an adjuvant setting (13), a recent meta-analysis by the Early Breast Cancer Collaborative Study Group failed to demonstrate a beneficial effect on disease free and overall survival of adjuvant tamoxifen in estrogen receptor (αER) negative patients (14). Consequently, the most recent 1998 Sankt-Gallen Conference Consensus advised against the use of adjuvant tamoxifen in αER negative patients, whatever their menopausal status.

Indications for tamoxifen treatment in patients with distant metastases include αER positivity, limited tumoral burden and the absence of life threatening conditions. Typical patients are those with soft tissue or bone metastases in the absence of liver involvement. If the hormonal receptor status is unknown, the choice for tamoxifen treatment is based on several prognostic factors such as age, disease free period, performance status and distribution and number of metastases.

Prediction to first line hormonal tamoxifen therapy in metastasised disease is based on αER measurements and the UICC (Union International Contre le Cancer) criteria. Since Jensens' work (15) correlated response to adrenalectomy with the αER status of neoplastic tissue, similar observations have been made for all available hormonal treatments : the presence of αER, especially in high concentrations is associated with high rates of responsiveness, whereas the absence of αER's or very low levels generally predicts failure (16,17,18). Most of the data on the clinical utility of αER content have been generated using biochemical ligand-binding assays (LBA's) (19,20,21,22), such as the dextran-coated charcoal assay (DCA). Since the first report of its independent prognostic significance almost two decades ago (21), the assessment of αER status using LBA's has been validated repeatedly and is generally regarded as the standard by which other methods are assessed. LBA's are, however, technically challenging, expensive and require a relatively large amount of fresh frozen tissue. The development of highly specific monoclonal antibodies and immunohistochemistry (IHC) techniques to localise αER's provided the potential to overcome most of the difficulties inherent to LBA's (23,24,25,26). Compared with LBA's, IHC is easier to perform, less expensive and applicable to a wider variety of samples. As with LBA's, the ultimate usefulness of αER status assessment by IHC resides in its ability to predict responsiveness to

therapy and clinical outcome. Most of the studies evaluating the clinical relevance of αER measurements by IHC have reported statistically significant relationships with clinical outcome. Direct comparison of these studies is, however, difficult as many different antibodies, detection systems with unequal sensitivities and specificities, and arbitrary calibrated definitions of αER positivity were used. Also, the majority of these studies evaluated patient populations of mixed clinical stage and treatment status, making it nearly impossible to separate the prognostic from the predictive implications of their results. Despite these largely unresolved issues, most laboratories today assess αER status almost exclusively by IHC on routine archival tissue samples.

Results of the role of αER , measured using either LBA's or IHC, as a predictive index of sensitivity to hormonal therapy have been summarized in several overviews (27,28,29,30). Whereas 60-70% of all breast cancers are αER positive tumours, only 50-60 % of these are associated with an objective response to ablative therapies. In contrast less than 10 % of the αER negative tumours are associated with objective response to ablative therapies. Thus, response to endocrine treatment can be predicted with an accuracy of 60-70%. While this is an improvement over random allocation, the issues of why 50 % of αER positive tumours fail to respond and yet 10 % of αER negative tumours do respond still need to be resolved. Potential causes of discordancy between αER status and therapy response include sampling errors, tumoral heterogeneity, alteration of the antiestrogen ligand or the αER receptor, and antiestrogen cellular efflux (31,32).

The UICC criteria used to define objective remission, stabilization or progression are in general based on morphological imaging and hence on volumetric changes, with a significant reduction in volume usually ensuing at the earliest 2-3 months following effective treatment. Specifically for assessment of response to treatment of osseous metastases the UICC criteria require sclerosis of lytic lesions as a sign of regression. However, some patients will have evidence of sclerosis in lytic lesions before starting therapy. Additionally, radiological evidence of response to successful therapy is often not evident for 6 months and may be delayed for more than 1 year (33,34).

Given the inaccurate predictive value of current αER measurements and the application of the UICC criteria, roughly 40-50 % of patients on first line hormonal treatment are deprived for at least 2-3 months of a potentially beneficial other treatment

option. Accordingly, better methods for predicting clinical response to antiestrogen therapy are needed. In this regard several radiolabeled steroidal and non-steroidal estradiol derivatives of which the tumoral uptake is believed to relate quantitatively to the content and binding characteristics of the αER receptor in the target tissue have been synthesized and their imaging potential and clinical usefulness evaluated in vivo in humans. This review is an attempt to place the available data in the perspective of recent pathophysiological findings.

II. ER RADIOLIGANDS FOR IN VIVO IMAGING

Over the last 20 years, several halogenated derivatives of estradiol, vynilestradiol (steroidal) and hexestrol (non-steroidal) have been synthesized (35-52). Few of these agents however reached the clinical stage (figure 1). These include substituents of estradiol halogenated at the 16β position and vynilestradiol derivatives halogenated at the 17-position, either in *cis* (Z) or *trans* (E) position . Most of these receptor radioligands (RRLs) fullfill the following three requirements : 1) high specific activity (radioactivity per unit mass of the RRL, > 1Ci/mmol), as receptors are saturable systems with limited uptake capacity in vivo,2) high receptor affinity with low non-specific binding, and 3) appropriate metabolic and clearance characteristics (53,54,55,56). Because of the high specific activity available with RRL's and the concern regarding excessive dosimetry to humans, *Kd* (dissociation constant) as a measure of affinity of the ligand to the receptor should be in the range of 0.1-50 *nM*. If affinity is too high (*Kd < 0.1 nM*), the rate limiting step becomes a measure of flow rate or membrane transport, rather than the ligand-receptor binding process. If affinity is too low (Kd > 50 nM), the signal to noise ratio may be significantly decreased due to the large amount of unbound free tracer in circulation and tumour visualization may merely reflect vasculature or blood volume in the tissue of interest (57). Low non-specific binding and appropriate metabolic and clearance characteristics are required to obtain optimal tumor to background ratios allowing depiction of tumoral lesions .

III. UPTAKE AND RETENTION OF ESTRADIOL AND RADIOLABELLED ESTRADIOL DERIVATIVES : PASSIVE INFLUX AND αER BINDING ?

Levels of estradiol range from 0.2-1.7 (mean: 0.4) pmol/g tissue and 0.2 - 0.5 (mean: 0.3)

pmol/g respectively in pre- and postmenopausal normal glandular breast tissue compartments of mastectomy specimens. Breast adipose tissue also displays levels of estrogens markedly in excess of those in peripheral plasma but these consist mainly of estrone. For many of glandular breast tissue samples, tissue to plasma ratio's tend to be higher than unity. In breast carcinoma, levels of estradiol tend to be higher than those found in normal breast tissue, ranging from 0.25 - 0.4 (mean: 0.7) and 0.15 - 4.0 (mean: 0.75) pmol/g in pre- and postmenopausal women respectively. Furthermore, the levels in blood circulation exceed those in plasma by varying amounts, the excess differing enormously and sometimes reaching more than 100-fold (58,59,60,61). This striking and enormous variation in concentration of estrogens between different specimens of breast tissue relates only in part to menopausal status and the differences between normal and carcinomatous tissue (60). The reasons for such high concentrations of estrogen in breast tissue have been subject to substantial investigation, the results of which point towards either active biosynthesis within the breast and/or active uptake of steroid hormones form the circulation against a concentration gradient.

In terms of uptake, Scharl et al. (63) using an indicator dilution technique that allowed monitoring of blood-tissue exchange of radioactivity in a continuous manner in anesthesized surgically prepared pigs with by-passed liver circulation. This technique showed rapid extraction of 125-iodoestradiol (IES) for most organs including the lungs, central nervous system, intestines and spleen …after intra-arterial injection in vivo. However, within minutes the organs released the 125-IES in its original chemical form back into the vascular system, with the exception of αER rich tissues and the kidneys which retained the 125-IES in its original form (although in the kidneys a nonpolar metabolite also accumulated). In humans, McNeil et al. (64) and James et al. (65), showed that following infusion of tritiated estradiol and estrone into patients both are taken up and concentrated within the breast. The accumulation was selective but variable, being more marked in breast cancer tissue than in normal tissue and especially associated with estradiol. Concordingly, Duvivier et al. reported on the existence of a positive arterial-venous gradient for estradiol in the blood irrigating mammary tumours (64-6).

The mechanisms by which this selective retention or uptake occurs are not well known. In general, estradiol uptake is believed to occur in a passive way with subsequent binding to αER's. Theoretically, the high degree of lipophilicity of

estradiol and radiolabelled derivatives could result in sequestration in adipose tissue and the plasma membrane of cells. Thus, the presence of significant amounts of adipose, stromal and epithelial tissue could partly account for the ability of breast tumours to retain estradiol or its radiolabelled derivatives. On the other hand, loss of either the stromal or adipose component of a tumour, or a reduced degree of vascularization, might be expected to also reduce a tumour's apparent ability to retain estradiol or its derivatives. This however does not explain the reduced accumulation in several in vitro cell line models and the high uptake of estradiol in tumoral tissue relative to a predominantly fatty tissue background. Retention of radiolabelled estradiol in estrogen target tissues through specific binding on the αER target was first reported by Jensen et al. (67). The number of αER's per cell is limited to approximately 10000 which translates to 1000 fmol (10^{-15} mol) receptor sites per mg of cytosolic proteins, corresponding *Kd* values are measured in the subnanomolar range (68,69). Recent discoveries ,however, suggest that in addition to αER binding, active membrane related sequestration through steroid binding protein receptors (SBPR), high affinity binding sites other than αER and intracellular estradiol depot formation are involved in the retention or uptake of estradiol .

Active membrane related uptake or binding :
Specific membrane receptors for sex steroid binding protein (SBP) were first described in human decidual endometrium and in hyperplastic prostate (70). Binding to these receptors, which are part of the G-protein receptor family is time-dependent, specific, saturable and occurs with high affinity (71). The SBP /SBPR interaction followed by the binding of estradiol to the complex protein/receptor causes a significant increase of the intracellular levels of cAMP (figure 2) and a significant reduction of both estradiol induced cell proliferation and estradiol mediated increase of progesterone receptors. The interaction, however, does not increase the amount of estradiol entering estrogen responsive cells (72) rather it "captures" an additional quantity of the hormone at the outer surface of cells. In humans, cytoplasmatic SBP, exclusively found in epithelial cell cytoplasm, is present in half of non-invasive breast carcinomas, particularly in those that are highly differentiated, and in 67.5 % of invasive tumors regardless of subtype and differentiation (73). SBP receptors are present in a higher percentage of αER positive tumours (75%) than in αER negative tumors (37%). Thus membrane sequestration of estradiol is likely to occur preferentially in hormone responsive tumors.

Non αER intracellular estradiol binding sites :

Monje et al. detected specific and saturable (^{3}H)17β-estradiol binding sites of high affinity (*Kd= 0.36nM*) in uterine rabbit microsomes at higher concentration than in cytosol (370 ± 98 vs. 270 ± 87 fmol/mg protein, respectively) (74). Use of monoclonal antibodies against the transactivation heat shock protein recognition and steroid binding domains of the nuclear estrogen receptor (67 kDa), revealed lower concentrations of the αER in membranes and the presence of five additional immunoreactive proteins of 57, 50, 32,28 and 11 kDa which were absent in cytosol. Moreover the antibody against the steroid binding domain was as effective as an inhibitor for cytosolic and membrane specific radioligand binding. Similarly, in humans, Lopes et al. (75) demonstrated the presence of microsomal estrogen binding sites (Kd = 0.1-0.6 nM) likely related to cytosol ER recycling. Furthermore these authors showed that the magnitude of estradiol binding to microsomes depends on the ER positivity of the tumors.

A second ER form , respectively βER, found to bind estradiol with a comparable *Kd* to αER was recently described (76,77,78). Its role in breast cancer aetiology and cancer biology, however, remains to be proven as available data suggest that the amount of βER relative to αER in breast carcinoma is minor and most studies on βER have been based upon mRNA, rather than protein expression (79,80,81,82).

Additional high affinity binding sites, other than αER and βER, but bearing ER characteristics were recently described by Masamaru et al. (83) Using osmotic minipumps to continuously infuse 3H-estradiol at rates producing serum concentrations ranging from pre- to postmenopausal levels for two weeks in oophorectomized Sprague-Dawley rats bearing nitrosomethylurea-induced mammary tumours, these authors were able to demonstrate a very high affinity, estrogen target tissue specific, estradiol binding component with a *Kd* two orders of magnitude lower (0.35×10^{-12}) than determined with standard in vitro techniques. A second estradiol binding component with the expected αER *Kd* in vivo of $1\text{-}10 \times 10^{-10}$ M was also present. Estradiol bound to both classes of binding sites could be displaced with DES within a 6hr period. Additionally, whereas tissue to blood ratio's were low in brain and muscle tissue (3:1), the ER-containing tissues, uterus and NMU-induced mammary tumors, concentrated 3H-estradiol with maximal tissue:plasma gradients of 346:1 and 32:1 respectively, when estradiol was infused at a rate of 1 ng/hr. However, when estradiol was infused at a rate of 750 ng/h, these gradients fell to 4:1 and 2.5:1 respectively, indicating that tissues concentrate estradiol to a greater extent at a low than at a high circulating estradiol level. Thus, it may be that the

actual binding estradiol affinity in target tissues in humans in vivo , especially at low estrogen concentrations, is much higher than usually estimated from standard, in vitro αER assays. Siiteri et al. (84) came to a similar conclusion regarding in vitro measurements of the estrogen receptor. Siiteri noted that the free fraction of estradiol present under in vitro conditions is underestimated because of the high concentrations of additional low affinity estradiol binders in tissue. Using isodialysis techniques to measure actual amounts of free estradiol under in vivo incubation conditions, Kd values for the αER were 2-fold lower than with the use of conventional in vitro methods.

Estradiol depots

In vitro, when incubated with estradiol, human mammary tumors have the ability to synthesize long chain fatty acids esters linked to estradiol at the 17-β position, the so called lipoidal estrogens (E-L). E-L remain exclusively in the cytosol and can behave as long acting estrogens because of their slow hydrolysis resulting in release of estradiol (85). The enzyme responsible for formation of E-L , fatty acylCoA: estradiol-17β acyl transferase, is present in both αER positive and αER negative tumours. Its contribution to estradiol retention however is likely low as suggested by available data presented by Adams et al. (86). These authors found that following exposure to $5nM$ tritiated estradiol, extensive and specific binding of estradiol to αER in MCF-7 and ZR-75-1 cell lines, occurred before appreciable synthesis of E-L was evident and the maximum level of E-L attained was only 3-9 % of the amount of estradiol bound to αER.

In summary, it is unlikely that uptake of radiolabeled estrogens merely reflects binding to αER and cellular uptake, sequestration and retention through other mechanisms, either membrane or non-membrane related, have to be considered. Additionally, given the discovery of the existence of very high affinity αER-like binding sites (Kd < 0.1 nM), and the potential for underestimation of αER Kd when using LBA's, part of cellular radiolabeled estradiol derivative uptake may reflect tumoral perfusion rather than binding to receptors.

IV IS THE AMOUNT OF RADIOLABELLED ESTRADIOL UPTAKE CONCORDANT WITH αER STATUS AND COULD IT BE USED TO PREDICT RESPONSE TO HORMONAL TREATMENT ?

Besides enhanced uptake from blood, in situ breast carcinoma estradiol synthesis in postmenopausal women contributes significantly to the high intratumoral estradiol concentrations found in about 70 % of breast carcinoma patients (87,88). Both in vitro and in vivo studies have shown that breast tissues have the capacity for estrogen biosynthesis and interconversion through respectively, aromatisation of androgens to estrogens and (predominantly) interconversion of estrone to estradiol (figure 3) (89,90,91). Although activities are low, they are comparable to those in other peripheral tissues and in vivo represent pmol amounts of estrogen being produced locally within the breast. The enzymes responsible for aromatisation and interconversion, respectively aromatase and sulfatase, have been detected in both αER-positive and αER-negative tumors (92,93,94). However, aromatisation and interconversion seem more likely to be of biological significance in αER-positive tumors. In both pre- and postmenopausal women, the activity of sulfatase is 50-200 times higher than that of aromatase (95,96).

Using dual isotope techniques, Reed et al. (97) showed that in postmenopausal women with breast cancer the extent to which endogenous estrogens derived either from uptake or from in situ production is very variable between different individuals. It seems that certain breast carcinomas obtain all estrogens from uptake, and others from in situ synthesis, wbut that the majority acquire estrogens from both sources. Consequently, no significant correlation between tumor in vitro aromatase or sulfatase activity and endogenous estrogens has been reported (98,99). In vitro as well as in vivo experiments suggest that both enzymes are under the control of estrogen in ER-positive cells, allowing protection of αER against excess estrogen (100,101). For instance, tumor cells deprived of estradiol adapt by increasing their level of aromatase, developing the capability to become more sensitive to estrogen precursors. Thus αER positive tumors displaying high local estradiol biosynthesis are likely to show a low estradiol uptake and vice versa. Finally, Yue et al. (102) demonstrated that tumors deprived of estradiol have the ability to adapt to estradiol deprivation to allow them to be responsive to four log lower amounts of estrogen than when studied under wild type conditions.

Thus it seems that the tumoral need of estradiol to effectuate a biological effect is highly variable intertumoral and can vary over time intratumoral, depending on local environmental conditions. The local estradiol needs are provided through both uptake as well as local biosynthesis ,which, in αER positive tumors are inversely related. Consequently, a lack of concordance between radiolabelled estradiol uptake and αER status is to be

expected and the predictive value of radiolabelled estradiol uptake for tamoxifen therapy response is likely to be limited.

Available data on imaging of radiolabelled estradiol derivative uptake in human breast carcinoma for measuring αER status in vivo are limited to halogenated estradiol 17β and vynilestradiol derivatives. Direct comparison of these data is not possible (table 1) owing to the use of different methodologies and cut-off values for measuring αER positivity, the use of both quantitative PET and semiquantitative SPECT, and differences in patient populations studied. Individual data, however, fail to substantiate a direct relationship between estradiol RRL uptake and αER status in keeping with pathophysiological findings.

Bromoestradiol : Using a pinhole collimator at 1 hr after injection of 150 mCi 16α-77Bromoestradiol-17β Katzenellebogen et al. were able to visualise DMBA-induced mammary adenocarcinoma in mature female Sprague-Dawley rats (103). The average uptake ratio in their series was 6.27 and tumour uptake could be selectively depressed (to an average ratio of unity) by treatment with excess estradiol. Biodistribution studies in the same rats showed that 16α-77Bromoestradiol-17β was predominantly cleared through the liver and intestines and to a lesser extent urinary. Extrapolation of rat data to humans, taking into consideration the slower clearance half-time and increased urinary excretion of estrogens in humans, suggested an effective whole body dose of 0.03 rad/mCi with the primary critical organs being the lower large and upper large intestine, dose received being 0.43 rad/mCi and 0.34 rad/mCi. In a small feasability study on eight patients, four of which suffered from primary and four from metastatic breast carcinoma, McElvany et al. found three abnormal scintigrams in patients with primary breast carcinoma, two of which were biopsied and found to be estrogen receptor positive. By contrast two patients with advanced tumours on antiestrogen therapy showed negative results and one abnormal scan was obtained in an αER positive patient with chest-wall metastases (104). No uptake of tracer was seen in the single αER-negative primary tumour. However, despite the use of a diverging high energy collimator, a low tumour to background contrast was obtained due to high scatter from the 521 keV peak (23 % abundancy) of activity residing in the liver, gallbladder and intestines.

Iodoestradiol : Given the identical in vivo behaviour in female Sprague-Dawley rats of 16α-77Bromoestradiol-17β and 16α-(125I or 123I) iodoestradiol-17β and the favorable imaging characteristics of 123I, subsequent studies were performed using 16α-123 iodoestradiol-17β (105). Preston et al. presented SPET data on 28 breast carcinoma patients acquired 2 h following the injection of 50 μCi/kg of 16α-123 iodoestradiol-17β (106). SPET imaging demonstrated eight of nine histologically proven breast cancers, four of which were ER positive, three ER negative and two of undetermined ER status. There was one false negative of unknown ER status. Four of four metastases and six of six known axillary nodes were visualised. Initially there were 12 "false-positive " axillary nodes and two "false positive" scans showing mediastinal involvement. However, with clinical follow-up, 2 of 12 axillae and 1 of 12 mediastina were found to contain cancer of sufficient volume to be detected by common radiographic techniques. More consistent results were reported by Schober et al. in a series of 21 women, 14 of whom were suffering from breast carcinoma and 7 from benign breast tumours (107). Following the injection of 111 MBq of 16α-123 iodoestradiol-17β and acquiring data up to 24 h pi., 7 out of 9 ER positive (> 10 fmol/mg tissue) tumors were visualised whereas all 12 ER negative , including the 7 benign diseases, were not. In 42 patients with known ER receptor status out of a group of 62 patients, an overall sensitivity of 66 % (20/30) and specificity of 92 % (11/12) was found by Scheidhauer et al. in a multicentre study using 16α-123 iodoestradiol-17. Tumour to non-tumour ratios in this series varied from 1.3 - 2.1(108) . Finally, in a series of 29 women undergoing diagnosis for primary or recurrent breast carcinoma, using 16α-123 iodoestradiol-17β Kenady et al. found that scintigraphic detection was most noteworthy in patients with chest wall tumors and inflammatory breast cancer (109). Agreement between readers was 98 % for true negative readings and 94 % for true positive readings, but only 60 % for false positive and false-negative readings. However, data on the sensitivity and specificity when compared to ER status in this series were lacking.

Fluoroestradiol : Because of the unique ability of PET to quantify tracer distribution in vivo, 16α-18 fluoroestradiol-17β (FES) was introduced as an agent for in vivo ER receptor imaging (110). Additionally, because PET provides substantially greater contrast than is possible with conventional nuclear medicine imaging techniques, its use with 18F offers potential diagnostic advantages. FES has shown specific activity, highly selective ER binding in vitro, and high affinity for ER-positive target tissues (eg. uterus and mammary tumours) in animal models (111). In clinical studies, FES has most extensively been studied as a radiopharmaceutical for breast carcinoma

imaging. Mintun et al. performed PET imaging in 15 patients suffering from primary breast cancer. In the nine patients in whom the αER status was known ,a correlation of 0.96 (p < 0.001) between (a) non-specific binding and partial volume effect corrected in vivo uptake of 18-FES expressed as percentage of the dose injected per mL x 10-4 and (b) αER concentration (fmol/ mg protein)(112). This high correlation, however, is likely an overestimation as the linear regression line in this series was fully determined by the two patients with the highest αER receptor concentration. The mean regional tumoral activity expressed as % ID/ mL.10^{-4} was 1.86 ± 2.21. In a subsequent study by the same group on 16 patients with clinical or radiographic evidence of metastatic disease, metastatic lesions within the regions imaged on PET studies were identified by other imaging studies in 14 out of 16 patients (113). The pre-therapy PET images with FES demonstrated accumulation of the radiopharmaceutical in the lesion or lesions of 11 of these 14 patients (79%) and in 53 of 57 lesions (93%). Two false positive findings were due to radiation fibrosis and an insufficiency bone fracture. Unfortunately, in vivo regional uptake values expressed as % ID/ml were not systematically available. Based on the available data in a small subgroup of 7 patients the correlation between pretherapy % ID/mL uptake vs. ER status (r = 0.36, p = 0.43) was much poorer than the initial results presented by Mintun et al. in primary tumors. The higher overall uptake values in this series (% ID /mL 10-3) is likely due to the later imaging (110 min pi. vs. 90 min in the series by Mintun et al.). Dehdashti et al. in a series of 53 patients (32 patients with primary breast tumours and 21 with clinical or radiological evidence of distant metastases or disease recurrence) found a good overall agreement 88% (35/40) between in vitro ER assays and FES-PET % ID/mL (114). It is of interest that all 5 cases of disagreement were positive on in vitro ER but negative on FES PET (SUV values < 1.0). Only one of these patients was treated with hormonal therapy and she did not respond. Finally, when multiple tumor sites were assessed in a given patient, concordance was present in 85 % of lesions which is in agreement with the level of concordance obtained using in vitro ER determinations. Mortimer et al. assessed the value of FES in women with breast cancer for predicting response to systemic therapy (115). Results of FES-PET were related to ER status. Cancers were considered functionally hormone sensitive if the SUV of the lesion on FES-PET was $\geq$ 1.0 and hormone resistant if FES SUV was < 1.0. In this series, all 20 ER- tumours were also FES-. However, of the 21 ER+ tumours, 16 were FES+ and 5 were FES-. Thirteen patients were treated with hormone therapy, and 8 (61%) responded to that therapy. Only 1 of the 5 patients whose tumours were ER+ but FES- received hormone therapy, and this treatment resulted in disease stabilization only. When compared with the in vitro assays of ER status, FES-PET had a sensitivity of 76 % and specificity of 100 %.

Vynilestradiol derivatives: Iodine (125I) labelled vynil derivatives of estradiol were first reported by Hanson et al. (116). Synthesis of such derivatives involves formation of the two stereoisomers : 20E and 20Z iodovynil estradiol. The most promising of both agents turned out to be the E stereoisomer, showing rat uterus to blood ratios of 19 and 16 at 1-2 h post injection, declining to 7 by 4 h post injection. Both agents however suffered from a high plasmatic protein binding which is likely the reason why human results with these agents were never reported. In contrast, 11β-methoxy iodovynil estradiols (MIVE) have a low plasmatic binding (117) . In a comparative analysis by Foulon et al. performed in 21-day-old mice, the trans-isomer (E) showed the highest affinity for αER's (Kd = 16 x 10-10 M) with uterus to blood ratios being 15-fold higher for this derivative (118) . However, as shown by Rijks et al. the cis- isomer has a higher uterus-to-fat ratio and a higher in vitro affinity for dimethylbenzanthracene-induced rat and fresh human mammary tumour ER (119). To date, for breast carcinoma imaging purposes, only the cis-isomer has been injected in humans. Cis- MIVE was injected into 19 women, 8 of whom were referred for initial evaluation of breast cancer and 11 for postoperative follow-up (118). Nine of the latter patients suffered from bone metastases. The primary tumor (size : 8-10 mm) was visualised in 2/4 pts with high αER concentrations (162-455 fmol/mg) and was not detectable in 4 pts with low αER concentration (6-32 fmol/mg). Axillary lymph node metastases were detected in 2 patients and bone metastases in 4 out of the nine patients with such metastases. Rijks et al. (120) reported on their results obtained using cis-MIVE in 25 patients, 12 with primary breast cancer and 13 with metastases evident from other imaging modalities. Planar imaging showed uptake in 11 of 12 primary carcinomas and in 9 out of 13 metastasised patients. αER IHC data available in 10 of the 12 primary cancers and in 11 out of 13 metastasised patients were concordant with cis-MIVE imaging in all 10 primary and in 10/11 metastasised patients. In a series of 13 patients reported by Nachar et al. who had been referred for cis-MIVE scintigraphy because of abnormal mammography or a suspect mass at physical examination, 5 out of 10 histologically proven breast carcinoma showed specific tracer uptake. In eight breast carcinoma patients a good agreement with in

vitro ER determination was found whereas in 2 patients scan results were false positive (121).

V CAN WE VISUALISE THE EFFECT OF TAMOXIFEN TREATMENT ON ESTRADIOL UPTAKE BY BREAST CARCINOMA?

Following the administration of tamoxifen, several extra- and intratumoral mechanisms are responsible for a reduction of estradiol uptake and intratumoral retention occuring in hormone-dependent, but not in hormone-independent, breast carcinoma tissue.

Extratumoral : In a series of 64 postmenopausal patients by Bertolissi et al. (122) an increase of 44 % (p = 0.000) in SBP concentration 30 days following the initiation of tamoxifen treatment was found, baseline values being 62.1 $\pm$ 3.3 nmol/L. Similarly, Rose et al. (123) demonstrated an increase in immunoreactivity of SBP blood concentration (p < 0.001) in 22 patients treated with tamoxifen for 3-36 weeks when first sampled, compared with 27 patients who were not receiving tamoxifen therapy. In the series by Rose et al., tamoxifen also produced a reduction in the percentage non-protein bound estradiol (p < 0.001) and percentage albumin-bound estradiol (p < 0.01), the two biologically available fractions, and a corresponding increase in the percentage SBP-bound estradiol (p < 0.01). As shown by Fortunati et al. the increase in estradiol bound SBP and its inability to bind to SBP receptors will result in a decrease of tumoral estradiol membrane sequestration. In postmenopausal women on tamoxifen treatment Lonning et al. further demonstrated a fall in plasma estradiol level (mean reduction of 11.9%, p< 0.05) possibly due to a reduced ovarian excretion of testosteron as evidenced by a mean fall in plasma FSH and LH values of 45.5 % and 48.1% (respectively p< 0.0001 for both)(124) .

Intratumoral : As shown by Pasqualini et al. and others, in hormone dependent breast carcinoma cell lines, tamoxifen and a variety of other molecules can block sulfatase activity (125,126,127).

In summary, tamoxifen treatment results in a decrease of absolute and free or bioavailable plasma estradiol as well as in an increase in SBP bound estradiol, resulting in a reduction of cellular uptake and membrane sequestration. Additionally, tamoxifen treatment decreases intratumoral estradiol synthesis resulting in a higher occupation of αER's by tamoxifen and consequently a reduced availability to the free fraction of estradiol. Theoretically, this should also result in a decreased uptake of radiolabelled estradiol derivatives following initiation of tamoxifen treatment initiation compared with baseline uptake.

Available data, although limited, support the usefulness of sequential radiolabelled estradiol derivative imaging for the purpose of predicting response to treatment as early as one week following treatment initiation, in keeping with pathophysiological data.

Dehdashti et al. (128) reported on the potential usefullness of consecutive FES imaging to predict responsiveness to antiestrogen therapy in patients with αER-positive metastatic breast cancer. Eleven postmenopausal women were included and underwent FES PET before and 7-10 days after initiation of tamoxifen therapy. There were seven responders and 4 non-responders on clinical follow-up (3-24 months). Responders had higher baseline SUV's when compared with non-responders ($\geq$ 2.2 vs. $\leq$ 1.7) and the degree of ER blockade by tamoxifen was greater in responders (mean SUV decrease 2.7 $\pm$ 1.7) than in non-responders (mean decrease 0.8 $\pm$ 0.5).

Comparable data were presented by Bennink et al. (129) using Z-MIVE to predict response or non-response to anti-estrogen treatment in metastasized patients. Among 23 patients treated with tamoxifen (40 mg daily) who underwent Z-MIVE scintigraphy prior to and 3-5 weeks after initiation of tamoxifen treatment, the 18 who showed complete blockage of αER activity responded to treatment. On the other hand, the 4 patients showing a mixed response and the single patient showing no blockage of ER showed progressive disease despite antiestrogen treatment. Patients with ER blockage had a significantly longer progression free interval (mean value $\pm$ SEM, 12.7 $\pm$ 1.7 vs. 1.6 $\pm$ 0.6 months, p < 0.001) (128).

VI CONCLUSIONS:

Available data in humans do not substantiate a quantitative relationship between uptake of estradiol radiolabelled agents and the content and binding characteristics of αER, in accordance with recent pathophysiological findings in breast carcinom**a.**

The tumoral need of estradiol to bring about a biological effect is highly variable intertumorally and can vary over time intratumorally, depending on local environmental condition. The local estradiol needs are provided through both uptake as well as local biosynthesis which in αER positive tumors are likely inversely related. Thus a lack of concordancy between estradiol uptake and αER status is to be expected, and the predictive value of estradiol uptake for tamoxifen therapy response is likely to be low, in accordance with available data.

In agreement with pathophysiological findings, early therapy prediction is likely feasible through rapid (prior to and one week following treatment initiation) sequential radiolabelled estradiol

scintigraphy, the accuracy of which seems higher than conventional αER measurements (cfr. Dehdashti et al. and Bennink et al.)

Additional significant advantages of radiolabelled estradiol scintigraphy as compared to conventional αER measurements which have to be taken into consideration are : 1) its non-invasive character and whole body assessment capability, 2) the fact that the receptor is not a biological product, which means that its production is not subject to animal variation as is the case in antibody production, 3) the more favourable biodistribution of the small labelled molecules which derives from their ability to often pass different biological barriers by mere diffusion, 4) the absence of errors of sampling and tissue manipulation as well as preparation and 5) the ability to address the intrinsic heterogeneity of receptor expression and estradiol uptake between the primary tumor and metastatic or recurrent lesions.

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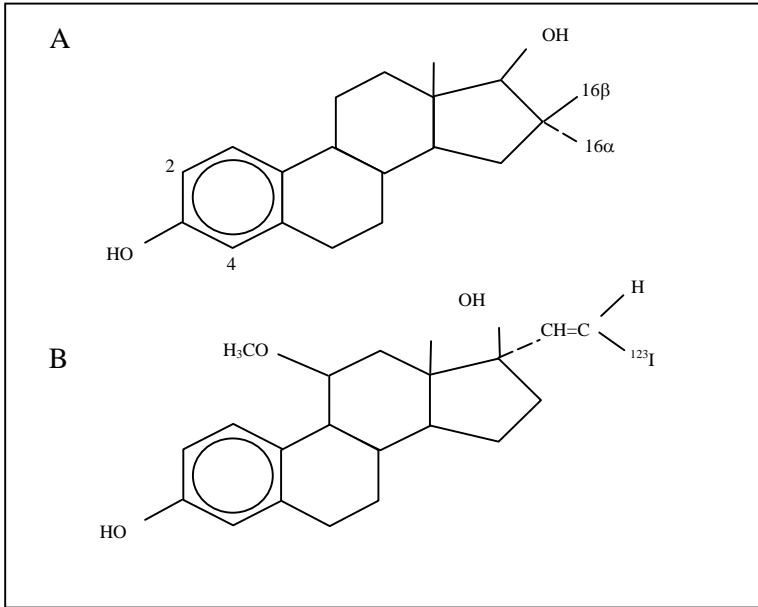
Figure 1. Structures of estradiol (A) and cis-methoxy iodovynilestradiol (MIVE) (B). Clinically used halogenated substituents for estradiol include 16a Bromo-, Iodo- and Fluoroestradiol.
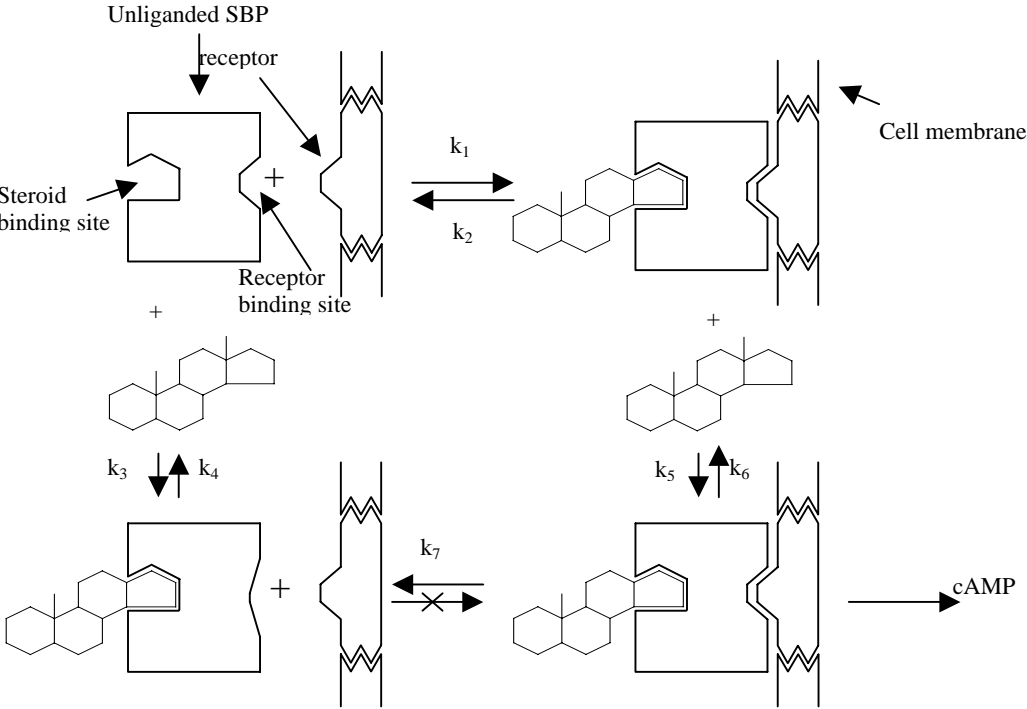
Figure 2.
Sex hormone binding protein (SBP) is an allosteric protein with two binding sites, one for steroids and the other for the SBP receptor (SBPR). Binding of a steroid to free SBP induces a change in the SBP membrane-binding domain that non-competitively inhibits SBP from binding to SBPR's. SBP/SBPR interaction followed by binding of a steroid to the SBP/SBPR complex however cause an increase in intracellular cAMP (Steroids 1999; 64: 100-106).
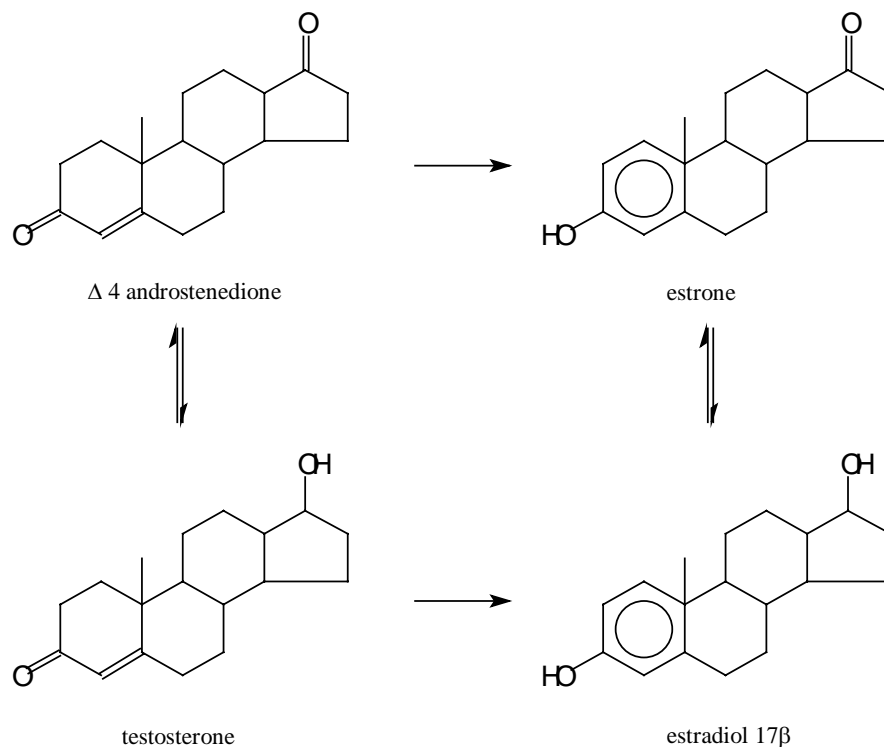
Figure 3.
Aromatisation of androgens (Δ 4-androstenedione and testosterone) to estrogens (estrone and estradiol).

Authors	Tracer	Nb of breast Carcinoma pts	M0/M+	ER status assessment	cut-off for ER positivity
McElvany et al., 1982	BES	8	4/4	LBA	≥ 10
Preston et al., 1990	IES	28	NA	NA	NA
Schober et al., 1990	IES	14	NA	LBA	≥ 10
Scheidhauer et al., 1991	IES	30	19/11	LBA	≥ 10
Kenady et al., 1993	IES	29	NA	LBA	≥ 3
Mintun et al., 1988	FES	13	13/0	LBA	≥ 3
Dehdashti et al., 1995	FES	53	32/21	LBA	≥ 3
Mortimer et al;, 1996	FES	43	26/17	LBA	≥ 3
Ribeiro-Barras et al., 1992	cis-MIVE	19	10 /9	LBA	≥ 6
Rijks et al., 1997	cis-MIVE	25	12/13	IHC	-/+
Nachar et al., 1999	cis-MIVE	10	10/0	LBA	≥ 9

BES = 16 α 77bromoestradiol , IES = 16 α 123iodoestradiol, FES = 16 α 18fluoroestradiol, MIVE = methoxy 123iodovynilestradiol, Nb = number, pts= patients,
MO = non-metastasised, M+ = metastasised, LBA = ligand binding assay, units are in fmol/mg cytosol protein,
IHC = immunohistochemistry, - = negative staining and + = positive staining.

Table 1.
Methodological and population characteristics of available data on radiolabelled estradiol derivative imaging in patients suffering from breast carcinoma.

BIODISTRIBUTION AND DOSIMETRY OF (123-IODINE)-IODOMETHYL-N,N DIETHYL-TAMOXIFEN : AN (ANTI)OESTROGEN RECEPTOR RADIOLIGAND FOR THERAPY EFFICACY PREDICTION

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ABSTRACT

This study reports on the distribution and radiation dosimetry of 123I-labelled trans Z-iodomethyl-N,N-diethyltamoxifen (123-ITX), a promising radioligand for prediction of therapy efficacy of unlabelled tamoxifen in human breast carcinoma. Whole body scans were performed up to 24 h after iv. injection of 123-ITX (μ:146 MBq, range: 142-148 MBq) in 5 female volunteers, four with and one without thyroid blockade. Blood samples were taken at various times up to 24 after injection. Urine was collected up to 24 h after injection allowing calculation of renal clearance and interpretation of whole-body clearance. Time activity curves were generated for the thyroid, heart, brain, breasts, liver and gallbladder by fitting the organ-specific geometric mean counts, obtained from regions of interest. The MIRD formulation was applied to calculate the absorbed radiation doses for various organs. The images showed rapid hepatobiliary excretion, resulting in good imaging conditions for the thoracic region whereas imaging of the abdominal region was impeded due to extensive bowel activity. The breast to non-specific uptake ratio increased over time. 123-ITX was cleared both by the kidneys and the gastrointestinal tract. At 50 h p.i. the mean excretion in the urine was 89.4 % (sd : 5.7%) . If the thyroid was not blocked, it was one of the critical organs. The highest absorbed doses were received by the excretory organs, i.e. the urinary bladder wall, the lower and upper large intestine, and the gallbladder wall. The average effective dose of 123-ITX was estimated to be 0.0084 mSv/MBq. The amount of 123-ITX required for adequate tumoral uptake imaging results in an acceptable effective dose to the patient.

INTRODUCTION

Estrogen deprivation therapy is effective in only 50-60 % of estrogen receptor positive (ER+) and in 5-10 % of estrogen receptor negative (ER-) advanced breast tumors (1,2,3,4,5). Therefore if potentially beneficial chemotherapy is not to be unnecessarily delayed it is important to be able to predict the likelihood of response to endocrine therapy prior to treatment initiation. By comparison with estrogen receptor status, the value of other markers eg. HER-2/neu, EGF-R, cathepsin D..., for this purpose is less proven and controversial (6). The reason why only 50 % of ER+ and yet 10 % of ER- breast carcinoma respond to estrogen deprivation is unclear and the causes of primary resistance remain to be resolved. Although treatment is typically successful initially, most estrogen dependent tumors will eventually resume growth, ultimately resulting in the death of the patient (7). Irrespective of whether resistance is primary or acquired, it is important to determine its cause (which is likely multifactorial) as a greater understanding of the mechanisms involved will lead to strategies by which resistance may be circumvented. In addition to the development of appropriate cell line models and xenograft systems (8,9,10), radiolabelled anti-estrogen derivatives that retain their anti-estrogen activity , allowing in vivo assessment of tumor uptake and washout, may facilitate progress in this area.

Yang et al. introduced trans-1-(4-(2-diethylaminoethoxy)phenyl)-1,2-diphenyl-5-mesyl-1-pentene (iodomethyl-N,N-diethyltamoxifen ,ITX) as an ER specific anti-estrogen for breast cancer imaging . In studies of human breast MCF7 breast tumor cell growth, MIC_{50} values were 11 μm for tamoxifen and 2.4 and 6.3 μm for the *cis* and *trans* isomers of ITX respectively (11). In mammary tumor bearing rats, ITX showed a tumor uptake value of 0.26 $\pm$ 0.17 % (injected dose/gram tissue). Upon priming tumor-bearing rats with estradiol, this value increased to 0.48 $\pm$ 0.11 at 6 hours, the mean estrogen receptor density as determined by the (3H) estradiol receptor assay being 7.5 fmol/mg protein. ITX scintigraphy of rabbits primed with diethylstilbestriol (DES, 1 mg/day, for 3 days, administered subcutaneously) clearly visualized the uterus which was no longer visible

after blocking with 15 mg DES. The uterus to background ratio was 9.6 (12).

Prior to 123-ITX injection in patients suffering from breast carcinoma, we studied its biodistribution and dosimetry in female postmenopauzal healthy volunteers as required by our local Ethics committee.

PATIENTS AND METHODS

Radiopharmaceutical synthesis

Trans-(123-I)iodomethyl-N,N-diethyl tamoxifen was prepared using a modified procedure described by Cherif et al. 20 mCi 123-I (13), dissolved in 40 μL 0.01 M NaOH, was added to trans-mesyl-N-diethyltamoxifen (1 mg) in 200 μL acetone. The reaction mixture was heated at 80 ° C for 20 min. After cooling to room temperature, the product was purified by RP-HPLC (ethanol : acetate buffer pH 5.0, 70:30 v/v , flow rate 1 mL/min, detection : UV 254 nm and NaI(Tl). The fraction containing 123-ITX (Rt = 13.0 min) was isolated, filtered through a 0.22 μm membrane filter into a sterile and pyrogen free vial and diluted with physiological saline up to 10 mL. The specific activity amounted to 5 $\pm$ 0.7 Ci/mmol. The radiochemical yield was approximately 65% and the radiochemical purity exceeded 98%.

Volunteers

This study was approved by the local ethics committee and performed according to good clinical practice. Five healthy, postmenopauzal female volunteers (mean age : 56 yrs, range : 50-61 yrs) were included in the study. To limit accumulation of free radioactive iodide in the thyroid following injection, with the exception of one patient (nb 1), the volunteers were pretreated with potassium iodide (10 % solution) orally, taken as two daily doses , two days before and one day after injection of 123-ITX (mean:146 MBq, range: 142-148 MBq) . The total dose of potassium iodide administered was approximately 300 mg. In the remaining patient (no. 1), the effect of no thyroid block was investigated.

Imaging

Volunteers were positionned supine with their arms alongside their body. Dynamic and whole body images were performed using a triple headed gamma camera (Irix, Picker, USA), equipped with low-energy high resolution parallel-hole collimators. The maximum useful peak energy of these collimators is 160 keV, with a corresponding septal penetration of 1.5%. A 15% energy window was centered at 159 keV.

Immediately following injection of 123-ITX, planar images of the abdominal region were recorded every minute during 30 minutes (matrix size 64x64). Whole body planar images were acquired 1, 2, 4, 6 and 24 h following injection in all patients, and up to 48 h for the first patient. Acquisition was performed simultaneously in anterior, posterior and left lateral position with a scan speed of 11 cm/min. Matrix size was 256x1024 pixels.

Blood sampling and urine collection

Blood samples were taken at 15s, 30s, 45s, 1m, 1m15s, 1m30s, 2.5m, 7.5m, 10m, 12m, 15m, 20m, 25m, 35m, 50m, 90m, 3h55m, 5h55m, and 23h55m following injection of the tracer. Urine was collected up to 24 h p.i. in three intervals, respectively from 0-6, >6-12 and >12-24 h p.i.. From each blood sample and the three urine collections, duplicate 1 mL aliquots were assayed for 123-iodine radioactivity in an NaI(Tl) counter (Cobra, Canberra Packards Instruments Company). Total blood volume and consequently activity was calculated using a total blood volume based on body weight and height (14). Total urinary radioactivity was determined by multiplying 0.5 mL urine activity by the total urine volume at each interval. Measurements were corrected for physical decay and the total amount of activity was expressed as a percentage of the injected activity (% IA).

Dosimetry

Image analysis

For quantification of radioactivity uptake after injection of 123-ITX, ROI's over the total body and organs of interest were drawn on the earliest images and the shapes and sizes. i.e. number of pixels , were kept constant over all subsequent images. Correction for non-specific uptake was performed by using a region over the shoulder. For each ROI, i.e. each organ, the geometric mean ,corrected for physical decay, of total anterior and posterior counts was calculated. The total body geometric mean activity, calculated on the first image (1 h p.i.) was taken as the total injected activity, considering that no urine was excreted prior to the first whole body scan. The activity in the total body and different organs was expressed as the percentage of the injected activity (% IA) calculated by the following equation : (geometric mean counts in organ or total body)/(geometric mean counts in first total body) x 100.

Dosimetric calculations

For each individual , time-activity curves were generated for the thyroid, heart, breasts, liver and whole body. Two routes of excretion were considered, i.e. urinary and faecal. Urinary bladder and intestinal residence times were estimated using the dynamic bladder model of Cloutier et al (15) and the gastrointestinal kinetics model as adopted in the ICRP 30 report (16). Activity leaving through the kidney-bladder pathway was assumed to accumulate in the urinary bladder with a voiding interval of 4.8 h (representing a rate of 5 times a day, typical for a normal adult). Of the activity excreted by the liver, 30 % of the activity was assumed to flow into the gallbladder, which empties its contents every 6 h in the small intestine. Activity in the intestines was assumed to pass through the various segments of the GI tract at standard rates, the mean transit time being 4 h for the small intestine, 13 h for the upper large intestine, and 24 h for the lower large intestine. As the specific uptake in organs and tissues was low, the accuracy of the 5 data points in time was not sufficient to enable a real compartmental model analysis with a reliable determination of the transfer coefficients. Instead, source organ residence times were determined accurately from integration of the best unweighted multiexponential fit to the experimental data. Target organ radiation doses were calculated applying the MIRD methodology (17) for the normal female adult using the MIRDOSE software package (18,19).

RESULTS

None of the subjects suffered from adverse events.

Whole body images of one subject showing the biodistribution of radio-activity upon injection of 123-ITX at different time points post-injection are presented in figure 1.

Dynamic imaging of the abdomen during the first 30 min following injection in two subjects showed a fast and early elimination of 123-ITX by the kidneys. Although the kidneys were not systematically visualized, there was activity in the urinary bladder, confirming prompt urinary excretion through the renal system. The assays of blood samples showed that the elimination of activity from the blood was initially fast. The total blood activity at 10 min p.i. was lower than 33 % of the activity measured at 1 min p.i. and progressively decreased thereafter. The whole body images obtained between 1 and 6 h p.i. show most of the activity to be distributed in the bladder, liver, gallbladder and intestines, reflecting the known urinary and hepatobiliary excretion of steroids. Uptake in the lungs and brain was low. Although 123-ITX is of potential interest for myocardial ER imaging, no significant myocardial uptake was seen. There was a diffuse uptake and retention of radioactivity in the normal breast tissue, with the % IA remaining practically constant during the scanning period. Uptake in the other oestrogen target tissues, the uterus and ovaries, could not be analysed due to overlying intestinal activity. Although they were of low quality due to low counting statistics, images acquired 24 h p.i. showed most of the remaining activity distributed in the intestines.

Biological radioactivity distribution data at various times after injection of 123-ITX and time-activity curves for the whole body, various organs and blood are presented in Table 1 and fig.2 respectively (the data are averaged over all five subjects and expressed as % IA).

The mean cumulative total measured urinary excretion at 6, 12 and 24 hours was 32.2% (SD 7.7%), 46.2% (SD 9.6%), and 60.8% (SD 9.4%) respectively (Table 2). The residence times calculated from the exponential fits for the various organs was highest for the remainder of the body in all subjects (n=5), followed by the urinary bladder (n=5) and the upper large intestine (n=4) (Table 3). In all subjects, the bulk of the activity injected was eliminated via the kidneys as evidenced by the calculated 50-day elimination of 89.4% (SD 5.7%). The mean radiation dose estimates (and standard deviation) calculated on the basis of the subjects' time-activity curves, are shown in Table 4. In the subject in whom the thyroid was not blocked, the thyroid was one of the critical organs, with an absorbed radiation dose estimate of 0.199 mGy/MBq. In the other healthy volunteers, the organs receiving the highest absorbed doses were involved in the excretion of 123-ITX. On average , the highest dose was received by the urinary bladder ($4.76^{E}-02 \pm 0.75^{E}-02$mGy/MBq) followed by the the lower large intestinal wall ($2.49^{E}-02 \pm 1.15^{E}-02$ mGy/MBq), the upper large intestinal wall ($2.00^{E}-02 \pm 0.90^{E}-02$ mGy/MBq), the gallbladder wall ($12.18^{E}-03 \pm 6.00^{E}-03$ mGy/MBq) and the small intestine ($6.85^{E}-03 \pm 2.55^{E}-03$ mGy/MBq). The dose received by the ovaries and uterus was $2.98^{E}-03 \pm 0.41^{E}-03$ mGy/MBq. The estimated mean effective dose for the female adult was $8.44^{E}-03 \pm 1.51^{E}-03$ mSv/ mBq.

DISCUSSION

For receptor tracers to be used in tumor imaging, three requirements must be met (a) high specific receptor affinity, with low non specific binding ; (b) high specific activity (> 1 Ci/ mmol), since receptors are saturable systems with limited uptake capacity in vivo ; and (c) appropriate metabolic and clearance characteristics. Early attempts to place an iodine atom on the aromatic ring of tamoxifen resulted in either low affinity or low specific activity analogues (20). Placing a chlorine atom on the aliphatic side chain of

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tamoxifen resulted in an analogue with a higher affinity than that of tamoxifen (21,22). Unfortunately, there is no existing cyclotron-produced isotope for chlorine. Replacing chlorine for a iodomethyl grou, however, yields a high specific activity analogue with a high binding affinity, 123-ITX (11,12).

Injection of 123-ITX in Sprague-Dawley and Fisher 344 rats showed high liver (6.764%+ 0.056% injected dose/g tissue at 1 h) and lung (2.835%+ 0.1596% injected dose/g tissue at 1 h) uptake as well as progressively increasing tumor to blood ratios reaching an optimum at 24h p.i (12). These findings imply that delayed imaging may be required in order to increase the 123-ITX tumour to tissue ratio and clear the high amounts of drug taken up by the liver and lung, allowing better depiction of 123-ITX uptake by tumours. The present study, however,shows low lung retention and rapid hepatobiliary excretion of the radiopharmaceutical, resulting in excellent imaging conditions for the thoracic region even at early time points (1h) post injection. The differences in biodistribution between rats and humans may de due to species differences in metabolism, e.g.a more rapid biliary clearance in man .

In addition to a predominant urinary excretion, 123-ITX was also cleared enterohepatically, resulting in a high accumulation of radioactivity in the abdominal region that impeded image interpretation. As 123-ITX is structurally similar to Chloro-tamoxifen α(Cl-TAM), the metabolic pathway of 123-ITX in the liver is probably dependent on the cytochrome P450 3A subfamilly-mediated demethylation and hydroxilation (possibly by an as yet undetermined cytochrome P450 isoenzyme), followed by glucuronidation and biliary excretion (23).

The MIRDOSE sotfware provides a calculation of the effective dose as defined in the ICRP 60 (24). Based on the effective dose of 8.44 E-03 (SD 1.51 E-03 mSv/ mBq) obtained in our study, both patients and volunteers could easily be investigated with 185 Mbq 123-ITX, allowing planar and single photon emission tomography imaging. The corresponding effective dose of 1.56 mSv is one third of the reported average effective dose per patient from nuclear medicine procedures in Europe (25). The dose received by the volunteers was significantly lower than the 5 mSv upper limit average effective dose of Category IIa of the World Health Organisation and Category IIb of the ICRP report(26, 27). From the distribution data for the four healthy female volunteers with the thyroid blocked, the mean radiation dose estimate for the thyroid was 4.70^E-03 mGy /MBq. In the subject without the thyroid blocked, the radiation dose received by the thyroid was 1.99^E-02 mGy/MBq, adding significantly to the effective dose equivalent. Thus blocking the thyroid will result in

a significant reduction of the radiation absorbed dose in clinical studies. Also, since the highest dose is received by the bladder wall, frequent voiding will reduce the absorbed dose to the urinary bladder wall.

The results of this study further demonstrate that 123-ITX is a pharmacologically safe radioligand (no adverse events were seen). The lack of a pharmacological effect was expected since only a tracer amount, 10 ng, was injected. This amount occupies only a small percentage of the human ER's.

In conclusion, the biodistribution of 123-ITX in five healthy female postmenopausal volunteers demonstrated low lung and liver uptake allowing early imaging of the thoracic region, with a favourable dosimetry.

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Table 1. Biological radioactivity distribution, expressed as percentage of the injected activity (%IA), in the total body, various organs and blood at different time points after injection of 123-ITX

Organ	Time points									
	1 h		2 h		4 h		6 h		24 h	
	AVG	SD	AVG	SD	AVG	SD	AVG	SD	AVG	SD
Total body	100	-	93.3	2.1	77.2	8.8	65.3	10.6	41.6	7.3
Heart	1.5	0.2	1.0	0.2	0.7	0.2	0.4	0.1	0.1	0.1
Liver	5.4	1.9	3.3	0.7	2.4	0.7	1.1	0.8	0.3	0.6
Gallbladder	2.1	1.6	1.9	2.0	1.3	0.8	0.7	0.6	0.3	0.4
Breast	0.5	0.3	0.4	0.3	0.4	0.2	0.3	0.2	0.1	0.2
Thyroid(a)	0.1	-	0.2	-	0.2	-	0.2	-	0.1	-
Blood (b)	20.9	4.2	16.1	3.1	12.2	1.3	6.1	3.3	0.5	0.2

a, thyroid activity for only one subject.
c, blood data are for five subjects at each time point.

Table 2 . Cumulative measured and predicted excretion fractions (%IA) for 123-ITX

	URINE				FAECES
	0-6 h	0-12 h	0-24 h	50 d	50 d
subject 1	26	40	54	86.2	9.7
subject 2	40	54	68	86.9	8.7
subject 3	41	58	73	83.0	9.1
subject 4	29	44	58	96.4	2.3
subject 5	25	35	51	94.3	4.7
mean	32.2	46.2	60.8	89.4	7.0
SD	7.7	9.6	9.4	5.7	3.1

Table 3. Residence times (h) for 123-ITX for each source organ

Organ	Subject						
	1	2	3	4	5	AVG	SD
Breasts	0.025	0.017	0.019	0.015	0.065	0.028	0.021
Gallbladder contents	0.045	0.047	0.032	0.012	0.086	0.044	0.027
Lower large intestine	0.505	0.505	0.574	0.146	0.219	0.389	0.193
Small intestine	0.320	0.320	0.364	0.093	0.139	0.247	0.122
Upper large intestine	0.619	0.619	0.702	0.179	0.268	0.452	0.269
Heart Wall	0.053	0.057	0.061	0.081	0.052	0.061	0.012
Liver	0.219	0.241	0.154	0.244	0.164	0.204	0.043
Thyroid	0.021	_	-	-	-	-	-
Urinary bladder contents	0.697	1.080	1.000	0.940	0.825	0.908	0.150
Remainder	11.200	8.780	8.100	10.900	10.100	9.816	1.341

AVG, Average ; SD, standard deviation

Table 4. Radiation absorbed dose estimates (mGy/MBq) for 123-ITX

Target organ	AVG	SD
Adrenals	2.98E-03	0.41E-03
Brain	1.83E-03	1.58E-03
Breasts	1.80E-03	1.25E-03
Gallbladder wall	2.18E-03	6.00E-03
LLI wall	2.49E-02	1.15E-03
Small Intestine	6.85E-03	2.55E-03
Stomach	2.98E-03	0.41E-03
ULI wall	2.00E-02	0.90E-02
Heart wall	4.10E-03	0.78E-03
Kidneys	2.98E-03	0.41E-03
Liver	2.34E-03	0.54E-03
Lungs	2.98E-03	0.41E-03
Muscle	2.98E-03	0.41E-03
Ovaries	2.98E-03	0.41E-03
Pancreas	2.98E-03	0.41E-03
Red marrow	2.98E-03	0.41E-03
Bone surfaces	2.98E-03	0.41E-03
Skin	2.98E-03	0.41E-03
Spleen	2.98E-03	0.41E-03
Thymus	2.98E-03	0.41E-03
Thyroid,b	4.70E-03	1.98E-03
Urinary bladder wall	4.76E-02	0.75E-02
Uterus	2.98E-03	0.41E-03
Total body	3.17E-03	0.34E-03
Effective dose equivalent,[a,b]	8.77E-03	0.93E-03
Effective dose,[a,b]	8.44E-03	1.51E-03

LLI, lower large intestine ; ULI, Upper large intestine
[a] Units of effective dose and effectiove dose equivalent are mSv/MBq
[b] Whole body effective dose , effective dose equivalent and thyroid dose were calculated excluding subject 1.
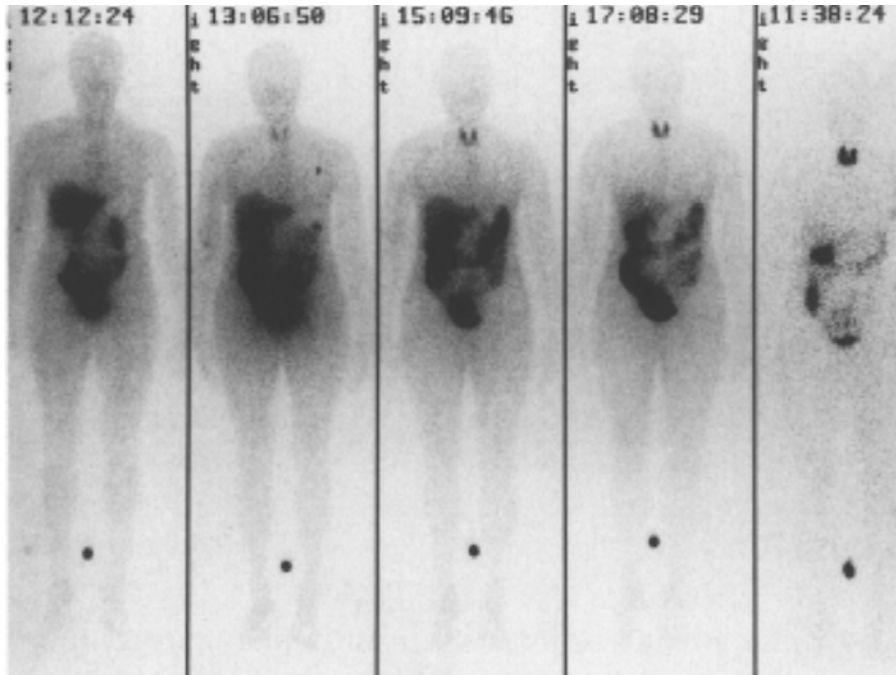
Figure 1. The anterior whole-body images obtained from subject 1 at 1, 2, 4, 6 and 24 h after intravenous administration of 123-ITX.
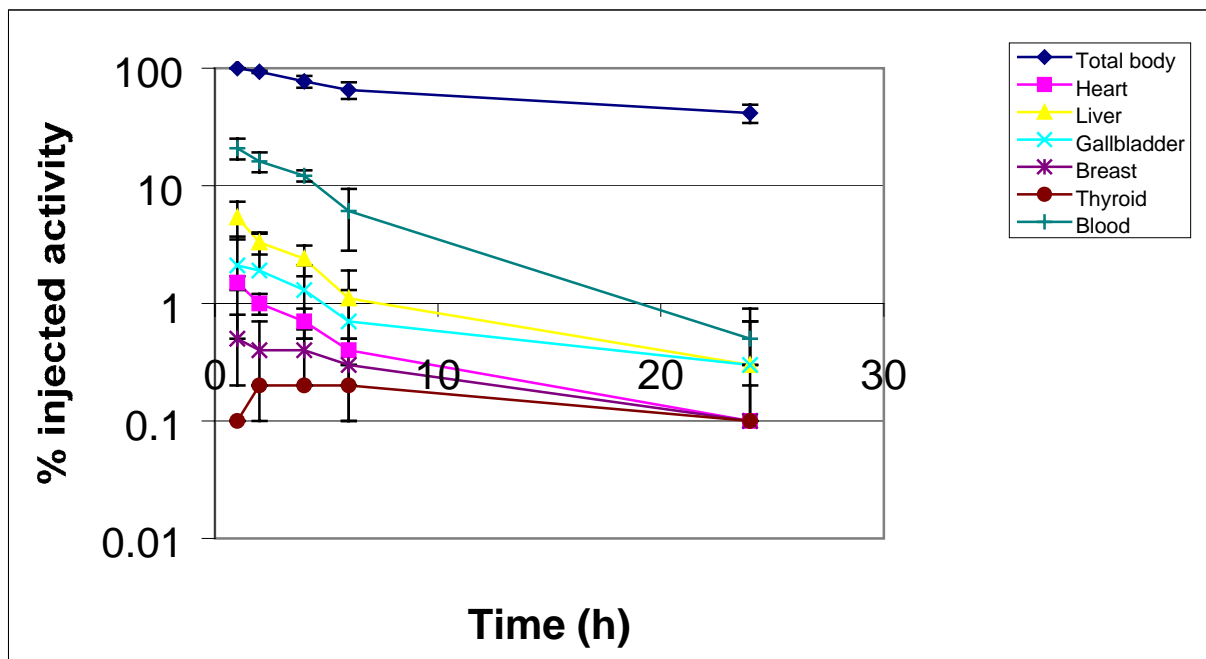
Figure 2. Time-activity curves for the total body, various organs and blood, calculated from direct measurements of counts in organ-specific ROIs or in blood. The physical decay-corrected data, expressed as % IA, are averaged over the five subjects, except for the thyroid (only one subject no.1). The error bars represent 1 SD.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 123-I LABELED TAMOXIFEN METABOLITES IN HUMAN PLASMA

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ABSTRACT

A high-performance liquid chromatographic procedure for the quantitation of [^{123}I]Iodomethyl-N,N-diethyltamoxifen (ITX), a radioligand for human breast cancer imaging, in human plasma is described. Separation was effected on a RP-C18 column, using a mixture of acetonitrile-water-triethylamine (70/30/0.5, v/v). ITX was rapidly cleared from human plasma and metabolites appeared as early as 7.5 min p.i. Quantitative assessment of metabolites in plasma over time allowed recalculation of the ITX plasma time-activity curve. Implications of ITX metabolite formation for breast tumour imaging are discussed.

INTRODUCTION

Although initially tamoxifen was earmarked for treatment of α estrogen receptor (αER) positive metastasised breast tumours with response rates of 40-50%, surprisingly and contrary to expectations up to 10% of patients with αER negative tumours also responded to tamoxifen therapy. The metabolism of tamoxifen with formation of various metabolites which may have different agonist/antagonist profiles is at least partially responsible for its in vivo capacity to achieve this effect. Unfortunately, in the course of treatment, sooner or later, all patients eventually develop resistance to tamoxifen therapy (2).

The issues of why 50% of αER positive tumours fail to respond and yet 10% of al αER negative tumours do respond as well as the causes of acquired resistance still need to be resolved (4). In addition to the development of appropriate cell line models and xenograft systems, radiolabelled anti-estrogen derivatives that retain their anti-estrogen activity, allowing in vivo assessment of tumour uptake and washout, may help to elucidate the causes of resistance to tamoxifen (3,5,8).

Trans-1-(4-(2-diethylaminoethoxy)phenyl)-1,2-diphenyl-5-mesyl-pentene-1 (iodomethyl-*N,N*-diethyltamoxifen, ITX) is an αER specific radiolabelled anti-estrogen for breast cancer imaging that has shown specific uptake in breast tumour cell lines and human tumours in vivo in both animals and humans (1,6,9,10). With the goal of optimizing the imaging protocol for depiction of ITX binding breast lesions and semiquantitative assessment of tumour ITX uptake, we investigated the clearance properties and metabolite formation of ITX in human plasma in vivo. This particularly as labeled metabolites not only complicate quantitative analysis but also provide an image background that can make it difficult to identify ITX binding lesions that are small or display low ITX binding.

METHODOLOGY

Chemicals

ITX was produced by reaction with n.c.a. Na[^{123}I]iodide and purified by RP-HPLC (1). Acetonitrile (HPLC quality was purchased by Lab-scan (Dublin, Ireland)). Triethylamine (analytical-reagent grade) was obtained from Sigma (Bornem, Belgium). All other chemicals were of analytical grade and purchased by Aldrich (Bornem, Belgium).

Equipment

The isocratic HPLC system consisted of a Shimadzu LC-8A pump (Shimadzu, Tokyo, Japan), an UV detector PU 4025 (Pye Unicam, Cambridge, UK), a syringe injector equipped with a 750-µL loop (Valco Instruments, Eke, Belgium) and a Waters 745 automatic integrator (Millipore, Waters, Milford, MA, USA). The eluate was collected in fractions with a Redifrac fraction collector (Pharmacia, Brussels, Belgium). All radioactivity counting was performed with a Cobra one-channel γ-ray spectrometer equipped with a 3 x 3 in. NaI(Tl) detector and an automatic sample replacer (Canberra, Meriden, CO, USA) (1in. = 2.54 cm). All radioactivity measurements were corrected for physical decay and geometry.

Chromatographic conditions

An Econosil 10-µm RP-C_{18} column (250 x 10.0 mm I.D.) (Alltech, Laarne, Belgium) was used. The mobile phase consisted of a mixture of acetonitrile/water/triethylamine (70/30/0.5 v/v). The flow rate was set a 5.0 mL/min. UV detection was achieved at 254 nm. The sensitivity of the UV detector was set at 0.5 A.U.F.S. A stock standard solution of ITX (0.1µmol/750 µL) was prepared in methanol and stored at ambient temperature.

Human plasma analysis

There was informed consent from all participants and the Medical Ethics Committee of the Ghent University Hospital approved all human experiments. Five healthy postmenopausal female volunteers (mean age: 56 years, range: 50 – 65 years) were injected i.v. with 148 MBq (range 141 MBq – 152 MBq) ITX by a rapid bolus injection. At different time points (varying between 15 sec and 2 h) venous blood samples were taken. Plasma was obtained by centrifugation at 1000 g for 3 min. A 500 µL plasma sample was transferred into an eppendorfcup and counted for radioactivity.

At four preset time points (5, 20, 35, 50 and 90 min post injection) 750 µL of acetonitrile was added to the 500 µL plasma samples. The mixture was vortexed for 1 min and centrifuged at 3000 g for 3 min. After centrifugation 1100 µL of the supernatant was separated from the pellet. Radioactivity was determined in the pellet and the supernatant. A 750 µL amount of the supernatant was resolved by the chromatographic procedure. The eluate was collected with the fraction collector at time intervals of 0.5 min and fractions counted for radioactivity. Blank plasma samples were spiked with 1 µCi ITX and treated identically.

RESULTS

Validation of the method

The retention time of ITX was 12.5 min. (k' = 4.0; hold-up time was determined with water). The extraction yield was calculated by the following formula:

$$\frac{Activity\ in\ 1100\mu L\ supernatant \times RSN(=1.2/1.1)}{Activity\ in\ 500\ \mu L\ plasma\ sample}$$

RSN is the correction factor for the 1100 µL of supernatant, which is isolated, compared to the total volume of 1200 µL after addition of acetonitrile.
The extraction yield of ITX from blank plasma samples spiked with 1 µCi was > 93% (S.D. 1.5%, n = 4). The stability of ITX during the extraction was controlled by HPLC. The radiochromatograms of the spiked blank plasma samples showed only one peak with the same retention time as ITX, indicating that no degradation took place during the extraction procedure. The relative proportions of unchanged ITX were calculated by the following formula:

$$\frac{amount\ of\ radioactivity\ containing\ ITX}{total\ amount\ of\ radioactivity\ injected\ on\ the\ column}$$

In all cases > 98% (S.D. 0.9%, n = 4) of the present activity remained as ITX. Control radioactivity measurements of the HPLC column, demonstrated that no radioactivity was retained by the analytical apparatus.

Human plasma analyses for ITX

The initial high concentration of radioactivity in the plasma (average 254 nCi/mL, S.D. 73 nCi/mL) between 1 and 1.5 min p.i. was followed by a rapid decrease, due to an immediate extraction of radioactivity from the blood by peripheral organs e.g. the liver. At later time points (between 7.5 and 35 min post injection) an increase of radioactivity in the plasma was observed in all volunteers (from 90 nCi/mL (S.D. 32 nCi/mL) to 128 nCi/mL(S.D. 8.5 nCi/mL)). The extraction yield for all plasma samples under these conditions was higher than 96%.
Fig. 1 shows an HPLC radiochromatogram (at 20 min p.i. of ITX) representative for the separation of ITX and its radioactive metabolite. The radioactivity in the plasma, represented by unchanged ITX decreased from 75% (S.D. 2.5%, n = 5) at 5

min p.i. to 19% (S.D. 1.8%, n = 5) at 90 min p.i. (Table 1 and figure 2). A logarithmic relationship between the amount of authentic ITX versus time was observed. The equation is expressed as:

$$\% \, I = 20 + 80 \times e^{(-0.08 \times time)}$$

This equation is used to correlate the time-activity plasma curve. The corrected curve could be fitted to an open, two compartment model with $T_{1/2}$ for the distribution phase of 0.85 min (S.D. 0.25 min) and $T_{1/2}$ for the elimination phase of 46.2 min (10.3 min). The equation could be written down as:

$$a1 \times e^{(-b1 \times t)} + a2 \times e^{(-b2 \times t)}$$

The individual values for a1, a2, b1 and b2 are shown in table 2. The corrected time-activity plasma curve for patient # 4 is shown in figure 3.

DISCUSSION

ITX was rapidly cleared from human plasma according to a two-compartmental model with polar metabolites appearing as early as 7.5 min p.i., dominating by 20-90 min p.i. As shown by Jacolot et al., the metabolisation of tamoxifen occurs predominantly in the liver via cytochrome P-450 enzymes located in the microsomes (4). The major metabolic pathways for tamoxifen in humans involve demethylation, deamination and hydroxylation of key positions on the phenyl groups of tamoxifen (8). Demethylation of the tertiary amine results in the major metabolite accounting for 95-100% of all tamoxifen serum metabolites, namely N-demethyltamoxifen. As ITX is structurally similar to tamoxifen, it can be expected that ITX shares much of its pharmacokinetic behaviour. However, although the position of the CH$_3$I group does not interfere with the demethylation proces, the radioactive metabolite found in the volunteers cannot be N-demethyl ITX. As N-demethyl ITX is more polar than ITX its HPLC retention time should be reduced when compared to ITX which was not substantiated by our data. Although deaminated and hydroxylated ITX derivatives are theoretically possible, biodistribution and dosimetry data in humans showing visualisation of the thyroid over time point towards free iodine. As iodine is polar and negatively charged, with the exception of the thyroid, it has only limited access to the intracellular space. Furthermore it has no affinity for αER. However, it will increase background activity owing to its presence in blood and extracellular space e.g. abdominal due to liver and stomach uptake, as well as in the intracellular space in the neck, due to thyroidal uptake.

As previously demonstrated in the human biodistribution and dosimetry study on ITX (7), liver uptake of ITX is rapid. This may explain why metabolites appear in blood in significant quantities as early as 7.5 min p.i. Metabolites dominate by 20-90 min p.i. and the blood metabolite curve declines only slowly after 20 min. Given the early blood disappearance of ITX and the slow decline of metabolites, from the standpoint of imaging and quantifying ITX tumour uptake, delayed imaging is unlikely to yield better results than imaging starting at 15-30 min p.i.

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Table 1

Unchanged ITX (%) in human plasma at different time points after i.v. injection of 148 MBq ITX (n = 5 for all time points).

Time (min)	Unchanged ITX (%)	S.D. (%)
5	75	2.5
20	34	3.9
35	25	1.6
50	22	2.3
90	19	1.8

Table 2

Corrected time-activity plasma curve parameters for human volunteers after i.v. injection of 148 MBq of ITX, according to an open two compartment model.

	# patient 1	# patient 2	# patient 3	# patient 4	# patient 5
a1	729	694	1364	203	201
b1	0.76	1.11	4.21	0.96	31.8
a2	65.0	93.5	43.1	76.4	79.4
b2	0.017	0.029	0.011	0.023	
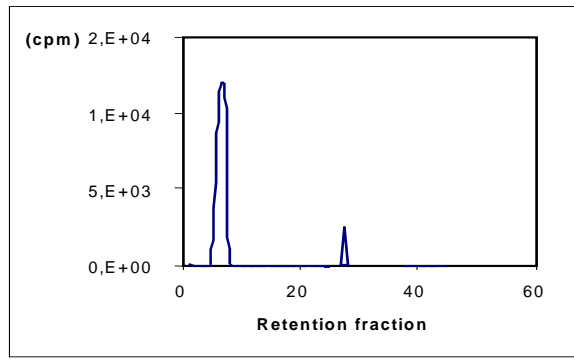
Figure 1

Representative HPLC radiochromatogram of a human plasma sample at 20 min after i.v. injection of ITX. The leftt

peak represents polar metabolites. The right peak represents original ITX.
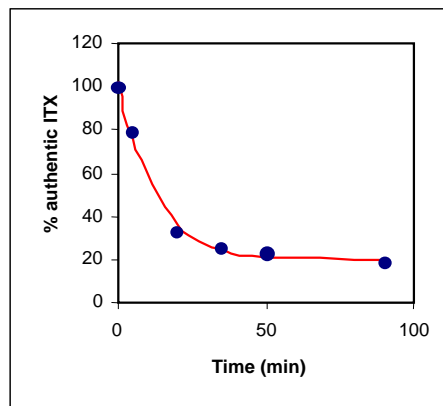
Figure 2

Amount of authentic ITX after i.v. injection of 148 MBq in humans. The curve represents the mean of five

patients. The fitted curve is also given.
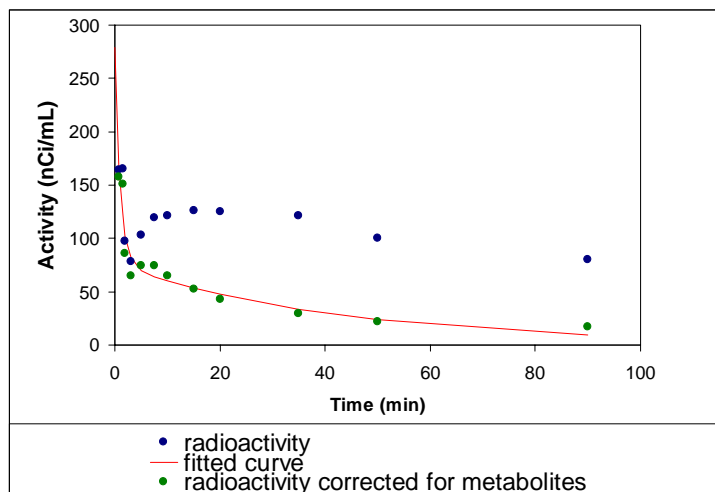
Figure 3

Time activity plasma curve of ITX versus time in patient 4. The corrected values for the amount of authentic ITX

are also given.

IODINE-LABELLED TAMOXIFEN UPTAKE IN HUMAN BREAST CARCINOMA

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ABSTRACT

As the mainstay of hormonal treatment for breast cancer patients (bcp) is tamoxifen (TAM), assessing uptake, retention and eventually efflux of 123Iodine-labelled tamoxifen (ITX) in vivo by means of scintigraphy could help to increase our understanding of tamoxifen's action and the mechanisms involved in resistance to the drug. This in turn could provide new targets for the design of therapeutic agents which can both effectively antagonise the alpha-estrogen receptor (α-ER) dependent growth pathway, as well as circumvent or prevent the emergence of inevitable resistance to the drug.

17 bcp were included in the study and divided in 2 groups. Group 1 (n, number of patients = 7) included untreated, histologially confirmed primary bcp, group 2 (n = 10) included TAM-resistant metastatic bcp, off hormonal treatment for at least 6 months. All bcp underwent whole body planar and tomographic (SPECT) imaging 30 min and 4-5 h pi. of 185 MBq ITX. Processed images were reviewed for the presence or absence of focally increased uptake at sites of known lesions from clinical or radiological findings. ITX uptake was quantified as the ratio of tumour lesion uptake to non-specific, contralateral breast or axillary uptake (T/N) on SPECT images. αER and progesteron receptor (PR) status was determined using immunohistochemistry.

In 4/7 bcp of group 1, all αER+ve/PR+ve, ITX uptake was clearly depictable. In 2 of these patients, involved axillary lymph nodes were also visualised. T/N ratios consistently increased over time. The remaining αER+ve/PR-ve and αER-ve/PR-ve tumours failed to show ITX uptake. In bcp of group 2, known tumour lesions in bone (n = 6), lung (n = 2) liver (n = 1) or liver and bone (n = 1) failed to take up ITX.

As PR positivity, reflecting αER functionality, in addition to αER positivity increases the likelihood of response to endocrine treatment, the lack of ITX uptake in αER+ve/PR-ve and αER-ve/PR-ve tumours may prove advantageous for predicting response to TAM treatment. The data presented further favor decreased intra-tumoral tamoxifen accumulation as a pathway for acquired resistance to tamoxifen in patients suffering from breast carcinoma.

INTRODUCTION

The precise mechanisms of resistance to tamoxifen and triphenylethylenes in general remain to be established. Based on currently available data, it seems most likely that the critical events driving response and resistance to tamoxifen are related to activities regulated through the alpha-estrogen signaling (αER) pathways.

The predictive value of breast tumour αER status, either assessed by ligand binding assays (LBA's) or immunohistochemistry (IHC) is well documented in both the adjuvant- and palliative setting (1-6). However, in the palliative setting, response to hormonal therapy is seen in only 50-60 % of αER positive patients with metastasized breast cancer and yet in 10 % of αER negative patients (4-6). This means that αER status is a good, but certainly not fully reliable parameter for predicting outcome to hormonal treatment in patients with breast carcinoma. While a loss of αER expression may not be the primary resistance mechanims to triphenylethylenes, when it occurs this loss is associated with acquired resistance. However, it appears that most tumours that acquire resistance in the clinic do so in the face of a continued expression of αER. Postulated mechanisms for triphenylethylene resistance in the presence of αER are (a) post-receptor alterations including changes in cAMP and the phosphorylation pathways,

or changes in coregulator and transcription factor interactions that affect the transcriptional activity of the αER, (b) changes in growth factor production/sensitivity of paracrine celle interactions or (c) pharmacological changes in the anti-estrogen itself, including altered uptake and retention or metabolism of the antiestrogen (7).

In vivo imaging of radiolabeled tamoxifen tumour uptake, retention and eventually efflux could help to increase our understanding of tamoxifen's action and the mechanisms involved in resistance to the drug. This in turn could provide new targets for the design of therapeutic agents which can both effectively antagonise the αER-dependent growth pathway , as well as circumvent or prevent the emergence of inevitable resistance to the drug in vivo.

This manuscript reports the first results of ITX (123iodomethyl-N,N-diethyl tamoxifen) imaging in patients suffering from breast carcinoma. In this feasibility study , using planar and SPECT imaging, primary and known metastatic, either resitant or non-resistant to hormonal treatment, breast tumor lesions in different types of tissue were investigated for the occurrence of accumulation of ITX. In previously untreated patients, ITX uptake was related to tumoral αER and PR receptor status as assessed by immunohistochemistry.

MATERIALS AND METHODS

Patients

Seventeen women (mean age : 54 yrs, range : 35-72 yrs) were included in the study.
Group 1 (table 1) consisted of 7 patients with a primary presentation of breast carcinoma, either proven by biopsy or strongly suggested by clinical and/or radiological findings, with clinically macroscopic disease. In all of these patients, histopathological diagnosis of breast cancer was established.

Group 2 consisted of 10 women suffering from metastatic breast cancer either proven by biopsy or strongly evident from clinical, radiological (computed tomography (CT), magnetic resonance (MRI), or ultrasound (US))and/or bone scintigraphic findings. All of these patients eventually acquired resistance to tamoxifen in the course of their treatment and were off tamoxifen treatment for at least 6 months at the time of imaging. Additionally, none of these patients had been recently treated by chemotherapy, or radiation therapy.

Informed consent according to institutional guidelines was obtained from all patients. The Ethical Board of the University Hospital Ghent approved of the application of the tracer to humans.

Radiopharmaceutical synthesis

Trans-(iodine-123)iodomethyl-*N,N*-diethyltamoxifen (ITX) was prepared using a modified procedure described by Cherif et al. 20 mCi ^{123}I, dissolved in 40 μL 0.01 *M* NaOh, was added to *trans*-mesyl-N-diethyltamoxifen (1 mg) in 200 μl acetone. The reaction mixture was heated at 80°C for 20 min. After cooling to room temperature, the product was purified by RP-HPLC (ethanol:acetate buffer pH 5.0, 70:30 v/v, flow rate 1 ml/min, detection: UV 254 nm and NaI(Tl)). The fraction containing 123-ITX (Rt=13.0 min) was isolated, filtered through a 0.22-μm membrane filter into a sterile and pyrogen-free vial and diluted with physiological saline up to 10 ml. The specific activity amounted to 5±0.7 Ci/mmol. The radiochemical yield was approximately 65% and the radiochemical purity exceeded 98%.

Imaging studies

All patients were pretreated with 300 mg potassium iode orally to block thyroid uptake of free radioactive iodide. Imaging was performed after a single intravenous injection of approximately 185 MBq ITX. For patients of group 1, the injection was given in the arm opposite of the known breast lesion to avoid false positive uptake in axillary lymph nodes. Patients were positionned supine, and at times of imaging of the thoracic region and breasts, with the arms raised alongside the head unless impossible due to physical constraints. Whole body images, planar spot images and tomographic images were performed using a triple headed gamma camera (Irix, Picker, USA) equipped with low energy high resolution collimator. The peak energy used was 159 kev, with a 15 % window. The usefull peak energy of the collimators used is 160 keV with a septal penetration of 1.5 %. For all patients, whole body images , spot images and SPECT (single photon emission computerized tomography) mages, were performed between 15-30 min and 4-5 h postinjection . Anterior and posterior whole body images were acquired simultaneously using a scan speed of 11.4 cm/min and a 256 x 512 pixels matrix size. SPECT images were acquired over 15 minutes by 40 views of 20 seconds per detector (60 angles; 3°angle; matrix size : 128x128). Transversal, coronal, and sagittal slices were reconstructed iteratively using OSEM (ordered subset expectation maximization, 6 subsets and 1 iteration) and postfiltered using a Butterworth filter (cut off frequency 0.8, order 7).

Data analysis

All studies were reviewed by two experienced observers familiar with the known normal biodistribution of 123-ITX in healthy volunteers. One of the observers was blinded to the clinical information and the findings of the other imaging modalities, but both were blinded with respect to the αER and PR status. Images were reviewed for the presence or absence of focally increased uptake at sites of lesions known from clinical findings and/or other imaging modalities, and for possible additional spots of uptake. Disagreements were resolved by consensus.

Quantification of abnormal 123-ITX uptake was performed using a region of interest method. ROI's were drawn over areas with increased 123-ITX uptake visualised on SPECT images ie. the tumors. In patients with primary breast carcinomas, the nonspecific uptake in breast tissue was estimated by drawing a similar ROI in the contralateral breast. For patients with metastatic lesions, nonspecific uptake of the radioligand was determined by assigning ROI's in visibly non affected comparable tissue. For each ROI, the average counts per pixel was calculated. For each lesion, the 123-ITX uptake was quantified as the ratio of total lesion uptake to nonspecific uptake (T/N).

In vitro αER and PR determination

In all patients, routine immunohistochemic assessment of αER and PR was performed on formalin-fixed paraffin-embedded tissue sections of the original primary tumor using commercially available antibodies.

RESULTS

Following injection of ITX, ± 1 nmol, none of the patients experienced adverse effects. Images showed low uptake and retention of ITX activity in the lungs in all patients. In keeping with the normal biodistribution data in healthy female volunteers, whole body images showed most activity distributed in the liver, gallbladder, and bowel, reflecting the hepatobiliary excretion of triphenylethylenes. Although there was no kidney-activity depictable, there was activity in the urinary bladder, suggesting prompt renal excretion of ITX. Low lung retention resulted in excellent imaging conditions for the thoracic region. Assessment of abdominal activity however was occasionally impeded due to bowel activity. There was diffuse and homogeneous accumulation of radioactivity in the normal breast. Uptake in the brain was low.

Patient data of group 1 are shown in table 1.

In 4 out of seven patients presenting with primary breast carcinoma ITX uptake was clearly depictable (see table 1 and figure 1) on SPECT images, but not always on planar images amongst others due to overlap with liver activity. All 4 patients suffered from αER+ve/PR+ve breast tumours. In two of these patients, involved axillary lymph nodes were also depicted on ITX imaging. T/N ratio's consistently increased over time. The remaining αER+ve/PR-ve and αER-ve/PR-ve tumours failed to show ITX uptake.

In patients of group 2, known tumour lesions either in bone (n, number of patients = 6), lung (n=2), liver (n=1) or liver and bone (n = 1) failed to take up ITX .

DISCUSSION

Yang et al. introduced ITX as an αER specific anti-estrogen for breast carcinoma imaging (8). In studies of human breast MCF 7 breast tumor cell growth, MIC 50 values were 11 μm for tamoxifen and 2.4 and 6.3 μm for the cis and trans isomers of ITX respectively. In mammary tumor bearing rats , ITX showed a tumor uptake value of 0.26 ± 0.17 % (injected dose/gram tissue). Priming tumor bearing rats with estradiol, this value increased to 0.48 ± 0.11 at 6hours, the mean estrogen receptor density as determined by the (3H) estradiol receptor assay being 7.5 Fmol/mg protein. ITX scintigraphy of rabbits primed with diethylstilbestrol (DES) (1mg/day, 3d, sc.) clearly visualised the uterus which was no longer visible after blocking with 15 mg DES. The uterus to background ratio was 9.6. Analysis of the biodistribution and dosimetry of ITX in female postmenopausal healthy volunteers demonstrated that ITX is pharmacologically safe and allows early imaging of the thoracic region, due to low lung and liver uptake, with a favourable dosimetry (9). This study reports on the uptake of ITX in patients suffering from primary untreated and metastatic tamoxifen resistant breast carcinoma.

Primary, untreated breast carcinoma

The majority of tamoxifen and its metabolites are bound to serum and only 2-5 % is in the "free" unbound state limiting the amount of bioavailable drug to the tumour (10). Nevertheless, reported intratumoural levels of tamoxifen and its metabolites following steady state are 5-7 fold greater when compared to serum or plasma levels, implying accumulation against a concentration gradient (11,12). In patients treated for less than 2 weeks with a dose of 30 mg tamoxifen /day, a significant

difference in the intra-tumoural tamoxifen concentration between αER+ve and αER-ve tumours was observed (450.1 + 75.3 ng/gm and 120.9+49.9 ng/gm, respectively; p = 0.04) (13). However, following at least two weeks therapy, approaching steady state conditions, no significant difference was found, with both αER+ve and αER-ve tumours accumulating high intra-tumoural concentrations. Thus, both αER+ve and αER-ve breast tumours progressively accumulate tamoxifen but αER+ve do so much more rapidly. The use of a bolus injection of ITX and consecutive imaging should allow for this difference in αER related uptake kinetics, with higher expected uptake values for αER+ve tumours, to emerge visually. However, as the apparent distribution volume for tamoxifen is + 50-60 liters/kg, implying extensive tissue binding related to its lipophilic characteristics, a high background activity may be anticipated (14). In this regard, in a study from patients receiving 40 mg/day for at least 30 days (ie. steady state), tamoxifen an its metabolites were found to accumulate in non-malignant human tissues 10-60 fold above serum levels expressed as ng/gm wet weight tissue to ng/ml serum (15). Nevertheless a depictable ITX uptake when compared to background activity was seen in 4 αER+ve/PR+ve patients but not in the remaining αER+ve/PR-ve and αER-ve/PR-ve tumours. As shown in the Early Breast Cancer Trialists meta-analysis, whereas αER positivity confers a 50 % response rate to front-line endocrine therapy, associated PR positivity, reflecting αER functionality, increases the likelihood of favorable response to endocrine treatment by 20-30 % (16). In contrast, patients who are αER and PR negative have a less than 5 % response rate to endocrine treatment. Consequently, the lack of discernible ITX uptake when compared to background activity in αER+ve/PR-ve tumours may prove advantageous for patient response prediction to tamoxifen treatment. Hypothetically, the lack of rapid ITX uptake in αER+ve/PR-ve may be explained by a lower affinity of tamoxifen to non-functional αER.

Till now, the precise mechanism for intracellular uptake and retention of tamoxifen and related derivatives such as ITX remains unclear. Given the high degree of lipophilicity of tamoxifen and ITX, passive diffusion similar to steroids may explain both breast tissue and tumour uptake as found in the study presented. However, this would not readily account for an apparent ability to concentrate ITX in tumour tissue relative to normal surrounding breast tissue. Currently, there are at least three major identified intracelullar binding compartments for tamoxifen : partition into cellular membranes, binding to high affinity αER's and binding to low affinity

antiestrogen binding sites (AEBS), membranous protein complexes intimately linked with the antiproliferative and antiretroviral effects of certain antiestrogenis compounds such as tamoxifen (17). As partition into cellular membranes is relatively aspecific, a similar degree of accumulation in primary breast tumours when compared to surrounding normal breast tissue is to be expected. Given the structural similarity between ITX and other AEBS binding tamoxifen derivatives, some of which 123I labeled, retention of ITX in tumour cells may also relate to the presence of AEBS. However, AEBS are low to moderate affinity binding sites (18) and depictable ITX retention through AEBS binding would require a steady state condition and not a bolus injection as performed in the series presented. Thus, increased tumoural ITX uptake when compared to the surrounding normal breast tissue in this series most likely reflects functional αER binding, in keeping with available animal data.

Metastatic, antiestrogen refractory breast cancer

Evidence in favor of reduced intra-tumoural accumulation as a potential pathway for acquired resistance to tamoxifen comes from both animal as well as human in vivo studies. In MCF-7 xenografts in immune-deprived mice which had become resistant following 4-6 months prolonged therapy, Osborne et al. observed a near 10-fold reduction in intratumoural tamoxifen compared with responding tumours in the absence of any change in serum concentrations (19). In a small clinical study from the same investigators, reduced intra-tumoural tamoxifen concentrations were found in 8/11 ""non-responsive tumours" treated with tamoxifen for between 1 month and 6 years, although serum tamoxifen levels were not measured to exclude poor compliance (20). Johnston et al. studied intra-tumoural tamoxifen accumulation, matched to serum concentrations, in a total of 51 tamoxifen-resistant tumours (21). There was no difference in the serum concentrations of tamoxifen or N-desmethyltamoxifen in patients who developped either acquired tamoxifen resistance after a median of 24 months, or were de novo resistant to the drug after a median of 4 months, or relapsed during adjuvant therapy after a median of 28 months. However a significant reduction in intra-tumoural concentration of tamoxifen in patients with acquired, but not de novo, resistance to drugs was found. In some patients even a ten-fold reduction in the ratio of tumour to serum tamoxifen levels was found, a quantitative difference similar to that observed by Osborne et al. In keeping with these findings, none of the hormone-resistant metastasised patients showed depictable tumoural ITX uptake when compared to normal

breast tissue. Although persistant occupation of αER's by tamoxifen, taking into consideration its long half-life in vivo, may partly explain this finding, its influence is likely to be low as all of the patients included were off antiestrogen treatment for at least 6 months.

Study Limitations

Due to referral limitations, no patients in whom tamoxifen treatment was going to be initiated because of metastasis consented to participate in this study. In these patients, it would have been interesting to relate the degree of ITX uptake prior to treatment initiation to the response to tamoxifen.

Conclusion

The preferential ITX uptake seen in αER+ve/PR+ve primary breast carcinoma suggests ITX holds potential for predicting response to hormonal therapy. The data presented favor decreased intra-tumoral tamoxifen accumulation as a pathway for acquired resistance to tamoxifen in patients suffering from breast carcinoma.

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human breast cancer and reduced intra-tumoural drug concentration. The Lancet 1993; 342: 1521-1522.

Table 1.

Age	TNMstage	Histology	αER	PR		early T/N	late T/N
65	$T_4N_1M_1$	ductal ca.	98%	5%		-	-
54	$T3N_2M_0$	ductal ca.	-	-		-	-
51	$T_2N_1M_0$	lobular ca.	95%	50%	T	3.7	5.0
					N	2.1	2.4
66	$T_3N_1M_0$	lobular ca.	95%	60%	T	1.8	2.0
					N	1.6	2.7
56	$T_3N_1M_0$	lobular ca.	100%	70%	T	1.2	1.6
					N	-	-
52	$T_2N_1M_0$	lobular ca.	80%	10%		-	-
45	$T_3N_1M_0$	ductal ca.	90%	90%	T	1.5	1.5
					N	-	-

Clinical, pathological and *trans*-(iodine-123) iodomethyl-*N,N*-diethyltamoxifen (ITX) single photon emission tomography quantitative results in patients of group 1.

TNM stage = clinical Tumour Node Metastases stage, αER = α estrogen receptor, PR = progesteron receptor, early T/N = Tumour to Normal tissue ratio's obtained 30 min post injection, late T/N = Tumour to Normal tissue ratio's obtained 4-5 h post injection.
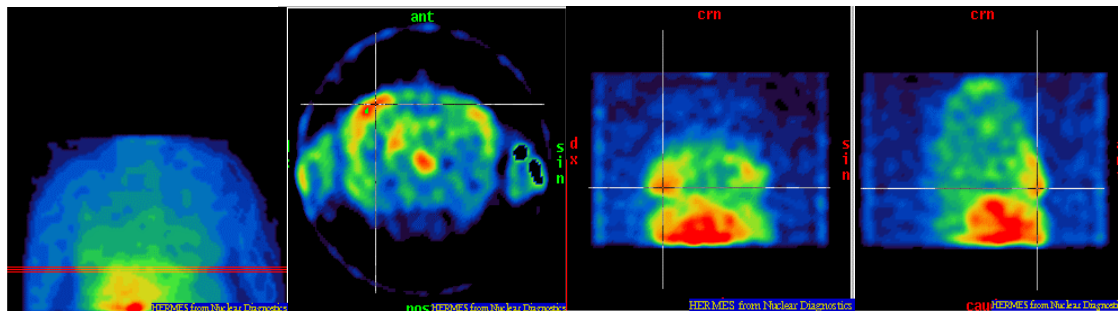
Legend to the figure

Figure 1

Imaging of breast cancer with trans-(iodine-123) iodomethyl-*N,N*-diethyltamoxifen (ITX).
ITX uptake in the primary breast tumour of a 66-old patient suffering from $T_3N_1M_o$ breast carcinoma disease as visualized by single-photon emission tomography (transaxial,sagittal and coronal slice) performed 30 min after injection of ITX. Physiological uptake in homolateral and contralateral non-involved breast tissue as well as persisting (intracardiac) bloodpoolactivity is also visualised.

PART 2 : GASTRIN RELEASING PEPTIDE RECEPTOR IMAGING IN BREAST CARCINOMA

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IS THERE A ROLE FOR AGONIST GASTRIN RELEASING PEPTIDE RECEPTOR RADIOLIGANDS IN TUMOUR IMAGING?

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I INTRODUCTION

The largest class of receptors encompasses proteins that consist of a single polypeptide chain of variable length that traverses the lipid bilayer seven times and utilises heterotrimeric GTP-binding proteins to govern effector proteins. Unravelling of the role of these receptors in proliferative signal transduction, their overexpression in carcinoma and their oncogenic potential as revealed within the past decade has led to the development of novel selective anticancer drugs (1,2). Simultaneously, several radioligands have been developed for in vivo imaging of these G-protein coupled receptors.

This manuscript reviews the rationale behind the development of agonist radioligands for visualisation of G-protein coupled gastrin-releasing peptide/bombesin receptor expressing tumours.

II BOMBESIN LIKE PEPTIDES AND GASTRIN RELEASING PEPTIDE

The tetradecapeptide bombesin was first isolated in 1971 by Anastasi et al. from the skin of the European discoglossid frogs bombina bombina and bombina variegata variegata using methanol extraction (3). The wide biological activity of bombesin and related peptides in rodents and amphibia e.g. gastrointestinal, renal and cardiac prompted the search for mammal bombesin (4,5) .

As the first mammal bombesin isolated from porcine non-antral stomach stimulated the release of gastrin in vivo, in spite of its numerous other physiological activities, the peptide was termed gastrin releasing peptide (GRP) (6,7). The human GRP equivalent was cloned and characterised five years later by Spindel et al. in 1984 (8). Naylor et al. pointed the single human GRP gene on chromosome 18 (9). As shown by Hildebrand, the biological potency of human GRP is similar to that of bombesin (10).

In humans and other mammals, GRP and bombesin are found predominantly in pulmonary neuroendocrine cells of foetal lungs, the neurones of the central nervous system and gut myenteric plexuses and to a lesser extent in prostate, <5 % of the total neuroendocrine cells, breast tissue and seminal fluid (8,11,12,13,14). Aside from its physiological role, GRP has been shown to be a tumour growth stimulating agent for a number of normal- and human cancer cell lines, grown in culture or as xenografts in nude mice. In these tumour models, the tumour growth effect is a direct result of binding of GRP to membrane G-protein coupled GRP receptors (GRP-R) on the cell surface, first isolated in 1990 (15, 16,17,18,19).Agonist binding to the extra-cellular face of the GRP-R results in activation of a guanyl nucleotide regulatory binding protein (G-protein) due to a change in conformation, resulting predominantly in activation of phospholipaseC$_\beta$ and occasionally adenylyl cyclase(20,21,22,23,24,25). Similar to other G-protein coupled receptors, for GRP-R, both homologous or agonist-specific- as well as heterologous or non-agonist specific receptor desensitisation are discriminated (26,27,28,29). Whereas prolonged administration of GRP or bombesin to GRP-R expressing cell lines results in receptor downregulation, pre-incubation with 1000 nM cholecystokinin (CCK) or with 1000 nM glucose-dependent insulotropic peptide (GIP) induce GRP-R upregulation(28,29,30).

The intracellular distribution of GRP-R is determined by four main pathways of movement of receptors within cells (31,32,33,34). Newly synthesised receptors are delivered to the cell surface from the Golgi complex. In unstimulated

cells there is a slow endocytosis of receptors from the surface into endosomes. In the presence of agonist, the rate of endocytosis is increased dramatically (35,36,37). The amount of agonist ligand endocytosed following receptor binding depends on the rates of ligand-receptor dissociation and hence ligand affinity and receptor endocytosis. Once the receptors reach endosomes, they can either be recycled back to the plasma membrane or routed to lysosomes for degradation. The ligand however remains in the perinuclear space where it is trapped in lysosomes (figure 2) (38,39,40,41,42). Specifically for high-affinity radiolabelled agonists, this entrapment will prolong the residence time of radioactivity in the target tissue when compared to antagonists, resulting in a higher accumulation of radioactivity in GRP-R positive tissues .

IV BOMBESIN-LIKE PEPTIDES AND HUMAN CARCINOGENESIS

During the past two decades, the role of GRP and its interaction with GRP-R as a tumour growth stimulating pathway has been studied in a wide variety of human cell lines, either grown in vitro or in vivo as xenografts in nude mice. Data on GRP expression and specifically GRP-R in human tumours however are limited to lung-, prostate-, breast-, colorectal- and gastric carcinoma. The latter will be discussed.

Lung carcinoma :
Cell line data: GRP/ bombesin-like immunoreactivity has been described in small cell lung carcinoma (SCLC)- and carcinoid cell lines but not in non-SCLC cell lines (43,44). As shown by Moody et al. SCLC cells secrete GRP/bombesin-like peptide into the surrounding media (45). Carney et al. and Weber et al. respectively demonstrated that bombesin and GRP act as potent mitogens on SCLC but not squamous- or lung adenocarcinoma (46,47). High affinity receptors for bombesin/GRP like peptides on human small cell lung carcinoma cell lines were first demonstrated by Moody et al. (48). As shown by several authors, the tumour growth stimulating effect of GRP on SCLC cell lines can be blocked by a wide variety of GRP antagonists (49-55).
Human data: The first evidence of bombesin-like immunoreactivity in human SCLC tumours was provided by Wood et al. showing high levels of GRP/BLI in a number of human SCLC tumours (57). In a larger and more recent series, using a broad panel of antibodies, Guinee et al. found bombesin like immunoreactivity in 45 % of open lung biopsy specimens (9/20)(58). The observation that GRP is specifically and actively secreted by SCLC cells and present in human SCLC resulted in the development of radioimmunoassays (RIA) and enzyme linked immunosorbent assays (ELISA) for

detection of serum pro-GRP from which GRP is derived through cleavage (59-60). Pro-GRP levels are increased in 76-79% of SCLC patients. Additionally, in this patient population proGRP is superior to Neuron Specific Enolase in terms of sensitivity, specificity and reliability and can be used for treatment monitoring and prognosis assessment (61,62,63,64,65)).

Using RT-PCR, Toi-Scott et al. found GRP-R expression in 17/20 SCLC cell lines derived from patients (66). The presence of GRP-R as well as GRP/BLP-like immunoreactivity and GRP secretion by human SCLC suggest GRP in these tumours may act as an autocrine growth factor. Evidence to support this hypothesis was recently provided by Kelley et al. Injection of 2A11, a murine monoclonal antibody which binds GRP-R with high affinity, at a dose of 250 mg/m^2 over 1 h three times per week for 4 weeks resulted in a complete remission in one and stable disease in 3 out of 13 previously treated SCLC patients. Of interest, human anti mouse antibodies did not increase during 2A11 administration in any patient and no toxic effects were recorded (67,68).

Prostate carcinoma :
Cell line data: GRP has been shown to enhance tumour growth, either in vitro or in nude mice, and invasion into matrigel in vitro of the GRP-R expressing prostate carcinoma cell lines PC-3 and DU-145. In both models, the GRP tumour growth stimulating effect could be blocked effectively by GRP-R antagonists such as RC-3095, RC-3095-II and RC-3940-II (69,70,71,72,73,74,75,76,77).
Human data: Using RT-PCR and in situ hybridisation (ISH), Bartholdi et al. demonstrated a widespread but variable presence of GRP-R mRNA expression in fresh frozen specimens of prostate carcinoma (12 cases) and benign prostate hypertrophy (6 cases)(78). GRP-R mRNA staining in cancerous tissue ranged widely from very intense to not detectable (about 30 % of cases), while normal tissue consistently displayed a low level of message staining. In a number of studies, the expression of neuroendocrine peptides including BLP's correlated with poor patient prognosis and hence associated with advanced metastasised and hormone refractory prostate carcinoma. Others, however, did not find a prognostic significance (79,80,81).

Data on GRP-R expression in prostate carcinoma are variable. Using ^{125}I-Tyr^4-bombesin as radioligand, Markwalder et al. detected GRP-R in 30 of 30 invasive prostate carcinomas and also in 26 of 26 cases of intraepithelial proliferative lesions, corresponding mostly to prostate intraepithelial neoplasias (82). Well differentiated carcinomas had a higher receptor density than poorly differentiated ones. Conversely, GRP receptors were identified in only a few hyperplastic

prostates and were localised in very low density in glandular tissue and, focally, in some stromal tissue. On the other hand, of 80 specimens of primary prostate cancer examined by Sun et al. using receptor binding assays, 50 (approximately 63%) showed high affinity, low capacity binding sites for bombesin/GRP (83).

Breast carcinoma :
Cell line data: Vangsted et al. and Yano et al. respectively demonstrated that bombesin stimulated growth of the human breast cancer cell lines T47D and MCF-7 MIII as well as of MDA-MB-231 (84,85,86). The growth effect in these cell lines could be suppressed by RC-3095, RC-3940-II or RC-3095-II both in vitro and in vivo in nude mice (85,86,87,88). In the MDA-MB231 cell line, Miyazaki et al. found a significant decrease in the number of binding sites for epidermal growth factor as well as for bombesin following chronic treatment, an effect which might be related to inhibition of tumour growth (87).
Human data : In a total of 41 human breast carcinoma biopsies from different menopausal patients, immunoreactivity against GRP was detected in 39 % of samples by Vangsted et al.(89). Similar results were obtained by Pagani et al. in a series of 28 primary breast carcinomas using Northern blot analysis with a pre-pro-GRP probe to measure GRP mRNA expression (90). In this series, moderate to strong expression of GRP mRNA was detected in 5 breast carcinomas whereas 4 other cases were weakly positive.

Halmos et al. examined the binding of (^{125}I-Tyr4)Bombesin to membranes isolated from 100 human breast carcinomas (91). Thirthy-three of these tumours expressed receptor levels of > 10 fmol/mg membrane protein, unrelated to patient age. Bombesin/GRP receptor expression was unrelated to patient age. Gugger et al. using ^{125}I-Tyr4-Bombesin and (^{125}I)D-Tyr^6, beta Ala^{11}, Phe^{13}, Nle^{14}-bombesin $^{(6-4)}$ found GRP receptor positivity to predominate in invasive ductal carcinoma (29/46 cases) and ductal carcinoma in situ (11/17) (92). Lymph node metastases (n=33) from those primary carcinomas expressing GRP receptors were all positive. GRP-R's were also present in high density but with heterogeneous distribution in normal ducts and lobules from all available breast tissue samples (n=23).

Colorectal carcinoma :
Cell line data: Frucht et al. studied 10 well characterised human colon cancer cell lines and found membrane bombesin receptors in 30 % (3/10) using ^{125}I-Tyr Bombesin (93). Radulovic et al. were able to inhibit growth of the human colon cancer cell line HT29 when grown as xenografts in nude mice using the GRP-R antagonists RC-3095 or RC-3440. The HT-29 cell line also displayed specific

binding sites for EGF which were consistently down-regulated following efficient treatment. Saurin et al. demonstrated that bombesin at a dose of 1 nM induced cell spreading and lamellipodia formation in the human GRP receptor positive coloncarcinoma cell line Isreco 1 (96). Ferris et al. provided evidence for a mutation-independent heptaspanning receptor constitutive activation resulting in cell proliferation of the non-malignant human colon epithelial cell line NCM160 and thus identified a potential mechanism whereby the GRP-R may act as an oncogene in humans (97).
Human data : Data on GRP and GRP-R expression in human colorectal cancer are conflicting. Preston et al. found 5 out of 21 colorectal cancers, but no uninvolved mucosa expressed high affinity, low capacity binding sites for GRP. Similarly, in a series by Radulovic et al. analysis of ^{125}I-Tyr4-bombesin binding data revealed the presence of specific binding sites in six (40%) of human colon cancer specimens (99). Using RT-PCR Saurin et al. detected widely variable GRP-R mRNA levels in 27/29 (93%) of tumour specimens (100). Significantly higher levels were found in poorly to moderately differentiated tumours (p<0.05) and in tumours with lymphatic vessel invasion (p< 0.01). Caroll et al. using IHC to detect both GRP and GRP-R, found 84 % of 50 randomly selected human resected colon cancers aberrantly expressed GRP or GRP-R, in ccontrast to only 1 of 37 metastases. Coexpression of both ligand and receptor was only observed in well differentiated tumours (62%), independent of disease stage suggesting that these proteins primarily act in vivo as morphogens. Kaplan-Meier analysis did not reveal any difference in patient survival between those tumours who did or did not express GRP/GRP-R. Finally, Chave et al. using receptor and ligand subtype-specific primers for RT-PCR as well as expression localisation by ISH, found GRP-R and ligand expression in colorectal cancer tissue as well as adjacent normal mucosa samples from 23 patients (102). GRP and GRP-R was present in greater expression in the tumour samples.

Gastric carcinoma:
Cell line data : Bold et al. found that bombesin stimulated growth of the human gastric cancer cell line SIIA could not be blocked with the GRP-R antagonists BIM 26189 and BIM 26226, but was inhibited by tyrphostin, a blocking agent of intracellular tyrosine kinases (103,104). SIIA cells possess GRP-R mRNA as measured by reverse transcriptase-PCR (105). Furthermore these cells possess an 80-kDa cell surface protein that specifically binds bombesin. Halmos et al. found high affinity ^{125}I-Tyr4-bombesin binding sites on crude cell membranes of MKN45 and Hs746T human gastric cancer grown in nude mice (106). In displacement studies, RC-3095 and RC-3950-II

dose-dependently inhibited 125I-Tyr4-bombesin binding. Treatment with RC-3095 at a dose of 20 µg/day decreased significantly the number of mitotic cells and serum gastrin levels as well as binding sites for epidermal growth factor (EGF) (107,108). Preston et al. described the presence of functional GRP-R's in the human gastric cancer cell line St42 (109).

Human data : Ferris et al. determined the normal distribution of GRP-R expression as determined by RT-PCR on cells lining the human GI tract obtained endoscopically by pinch biopsy (110) and found only cells lining the gastric antrum showed GRP-R expression implicating GI cancers aberrantly express GRP receptors. In a series by Preston et al. 13 of 23 gastric cancers expressed high affinity binding sites for GRP/bombesin of which 12 were subsequently characterised and found to be of the GRP-preferring subtype (111). Finally, Caroll et al. looked at the incidence and quality of GRP-R, as measured by the presence of GRP- receptor mRNA, aberrantly expressed by non-antral gastric adenocarcinomas, and evaluated the impact of receptor expression on patient survival (112). Overall, 8 out of 20 consecutive non-antral gastric adenocarcinomas (40%) aberrantly expressed GRP-R, 6 of which were found to be mutated. Pharmacologically, the effect of these mutations ranged from rendering the GRP-R non-functional to constitutively active. Contrary to expectations, survival of patients whose tumour expressed functional GRP-R (18.5±9.8 months) was not statistically different from those that did not (8.3±1.8 months; p=0.24).

DISCUSSION

The GRP/GRP-R interaction is an established tumour growth stimulating agent for several neoplastic human cell lines either in vitro or grown in nude mice. The mechanism by which tumour growth stimulation occurs does not appear to be unique but in general seems to involve transactivation and up-regulation of EGF receptors. In human tumours, the role of GRP/GRP-R is less clear. Over-expression of GRP-R's when compared to normal surrounding non-cancerous tissue has been described in SCLC, prostate-, breast-, colorectal- and gastric cancer. In these tumours, stimulation of GRP-R's may occur through paracrine produced GRP released by neuroendocrine cells present in e.g. human prostate and lung, particularly as GRP may diffuse several micrometers through tissue to exert it's effect on nearby cells. On the other hand, stimulation of GRP-R's may occur through tumour produced and released GRP. At present, simultaneous GRP/GRP-R co-expression has only been studied and described in colorectal carcinoma using both IHC and RT-PCR with conflicting results. Whereas

Caroll et al. using IHC found GRP/GRP-R in 62% of samples (31/50), Chave et al. using RT-PCR found GRP/GRP-R co-expression in 100% of samples studied (23/23) (101,102). Additionally, the incidence of GRP and GRP-R expression in individual reports looking either at tumour GRP-R or GRP also tend to vary significantly e.g. prostate- and breast carcinoma (82,83,91,92). Apart from differences in patient inclusion, this variation likely relates to differences in methodology used for GRP or GRP-R estimation, either IHC, mRNA analysis (RT-PCR or in situ hybridisation) or membrane ligand binding assays. All of these in vitro assays have some major drawbacks. Semiquantitative visual analysis of immunohistochemically stained tissue sections is operator dependent requiring both judgement and experience of the observer which renders reproducibility suboptimal. Although negative controls are generally used for standard immunohistochemistry, "specific binding" actually represents the "total binding" of the antibody to the tissue and non-specific binding cannot be measured directly. ISH, blotting and PCR-techniques are semiquantitative, labour intensive and require high sensitivity of the probes used. Additionally, blotting and PCR are applied to bulk tumour homogenates, which compromises the distinction of tumour cells from normal stromal cells, possibly expressing GRP or GRP-R. Importantly, the presence of mRNA for GRP or GRP-R is not synonymous to the presence of GRP or GRP-R. Lack of correlation between mRNA and peptide concentrations of GRP and GRP-R, indicating regulation at a post-transcriptional level has been reported in normal gastrointestinal tractus tissue and in vitro on several tumour cell lines. As for radioligand binding, fixation on prepared membranes does not imply the existence of functional receptors that become internalised following binding. Finally, all in vitro assays are dependent on sampling of tumour material.

Considering these drawbacks, a non-invasive, highly sensitive and specific technique to detect functional GRP-R and eventually GRP expression in vivo would be most welcome. Consequently, the development of radiolabelled bombesin analogues in nuclear medicine has attracted a considerable amount of interest in the area of cancer imaging and therapy. Taking into consideration the pharmacology of GRP/GRP-R interaction, agonists are preferable to antagonists as they are internalised, resulting in a higher accumulation of radioactivity in GRP-R positive tissues. Additionally, provided washout of internalised isotope or radioligand e.g. due to degradation does not occur, radiolabelled agonists may allow depiction of heterologous desensitisation through sequential imaging. In this setting, the absence of radioligand internalisation implies a

progressive decrease in membrane binding over time and thus an antagonist like behaviour.

V. RADIOLABELLED BOMBESIN ANALOGUES

Although radio-iodinated Tyr-containing peptides could easily be applied in vivo for receptor targeted scintigraphy and radiotherapy, these molecules are often rapidly degraded in vivo with release of radioiodine hampering their diagnostic and therapeutic use. With this regard, degradation of ^{125}I-Tyr4 Bombesin following internalisation with cellular iodine washout has been reported by several authors (36,113,114).

Currently, the most practical radionuclides for labelling peptides and other ligands are ^{111}In and ^{99m}Tc, both of which require the conjugation of chelating agents to peptides or other ligands. In recent years, a number of GRP analogues with delayed proteolysis and high potency have been developped. Baidoo et al. reported on the synthesis and initial evaluation of technetium diaminedithiolate analogues derived from the potent bombesin analogue Tyr-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$ (115). Results of in vitro competitive binding assays in rat brain cortex membranes against (^{125}I-Tyr^4)bombesin showed high affinity for these technetium analogues. Karra et al. using a new bifunctional chelating agent based on a tetradentate dithiadiphosphine framework (P2S2-COOH) synthesised ^{99m}Tc-P2S2-Bombesin(7-14) (116). Pharmacokinetic studies in normal mice showed fast and efficient clearance from the blood pool, excretion through the renal and hepatobiliary pathways and a significant uptake in the GRP-R expressing pancreas (pancreas/blood and pancreas/muscle ratios at 4 h pi. being ca. 22 and 80 respectively). Varvarigou et al. reported on the synthesis of Cys-Aca-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-met-NH$_2$, a ligand that showed high lung activity in mice as well as hepatobiliary excretion (117). Zinn et al. demonstrated specific, high binding affinity of the HYNIC chelated peptide Gln-Trp-Ala-Val-Gly-His-Leu-Met-CONH$_2$ to GRP receptors (118). Breeman et al. reported on the synthesis of a number of DTPA-bombesin analogues who were studied for their binding characteristics to the bombesin/GRP receptor on 7315b rat pituitary tumour cell membranes in competition with (^{125}I-Tyr^4)BN (119). Effects of bombesin analogues were determined on basal and bombesin-stimulated prolactin secretion by 7315b cells to distinguish between their agonistic and antagonistic characteristics. Of the DTPA complexes studied, the antagonist (DTPA-Tyr^5,D-Phe^6)Bombesin(5-13)NHEt and the agonist (DTPA-Pro^1,Tyr^4)Bombesin showed the highest affinity for the bombesin/GRP receptor on 7315b cell membranes. Despite similar affinity for the receptor, the ^{111}In-labelled agonist, but not the antagonist, was internalised by the bombesin/GRP receptor-positive tumour cells. Consonant with this observation, the agonist (DTPA-Pro^1,Tyr^4)Bombesin showed much higher specific uptake in bombesin/GRP receptor positive tissues and tumours than the antagonist (DTPA-Tyr^5,D-Phe^6)Bombesin(5-13)NHEt, with concordant target to background ratios. Biodistribution of this agent in rats showed high and specific uptake in tissues of the gastrointestinal tract , notably pancreas. Uptake of radioactivity was blocked by pre- or co-injection of 100 μg (Tyr^4)Bombesin, but not when this was administered 30 min after the radioligand. This finding suggests Bombesin/GRP-R mediated internalisation of the radioligand within 30 minutes. Specific uptake was found in receptor positive CA20948 pancreas tumour and CC531 colon carcinoma in tumour bearing rats. The CA20948 tumour, inoculated in the hindleg, was also visualised scintigraphically (120).

Hofman et al. reported on the synthesis of ^{99m}Tc RP527, a targeting peptide derived from bombesin, linked at its N-terminus via a linker group to a peptide sequence which chelates [99m]Tc. Both the unlabelled and labelled peptide bind GRP receptors with a similar affinity to bombesin (IC 50 value for displacement of ^{125}I-bombesin : 2-6 nM). As ^{99m}Tc RP527 is internalised by cells expressing the GRP-R it is believed to be an agonist. In SCID mice studies, ^{99m}Tc RP527 showed an uptake of 0.2-1.8 (μ), 0.0-0.6(SD) % ID at 4 h with a tumour to muscle ratio ranging from 9.8-33.7 at 1h and 6.5-25.4 at 4 h in human prostate (PC-3), pancreatic (CF-PAC-1) and rat pancreatic 5AR42J) tumour cells (121). Biodistribution and dosimetry analysis of ^{99m}Tc RP527 in humans showed rapid hepatobiliary excretion, resulting in good imaging conditions for the thoracic region, whereas imaging of the abdomen was impeded by extensive bowel activity. The highest absorbed doses were received by the excretory organs, i.e. the urinary bladder and gallbladder wall. The average effective dose of ^{99m}Tc RP527 was estimated to be 0.0095m Sv/MBq (122). In a small pilot study on ten patients suffering from prostate- or breast carcinoma, ^{99m}Tc RP 527 uptake was seen in 5 patients (123). Tumour to background ratios in these patients derived from planar and tomographic images increased significantly from 1.65 (SD : 1.53) and 3.35 (SD:3.04) to 2.58 (SD:1.26) and 7.23 (SD:8.46) at 1 h and 5-6 h pi. (p< 0.01 for both planar and tomographic ratios), due to a decrease in background activity. SPECT imaging showed consistently higher T/N ratios than planar imaging (p<0.001). None of the patients suffered from adverse or subjective side effects.

Safavy et al. reported on the synthesis of tris-bombesin conjugates with [188]Re . The yields of chemical syntheses ($\pm$ 20 %) and radiolabelling ($\geq$ 90 % yield) as well as the positive binding of the radiolabelled conjugates to GRPR-positive tumours reveal promise in the use of these molecules for cancer therapy (124).

VI CONCLUSION

The role of GRP/GRP-R on tumour promotion and growth is well established in vitro on human cell line cultures and tumour xenografts grown in nude mice. However, data on the role of GRP/ GRP-R in human malignancies are limited and vary significantly according to the in vitro methodology used for GRP or GRP-R detection. In analogy to the diagnosis of somatostatin receptor-expressing tumours in vivo with somatostatin receptor scintigraphy, in vivo GRP-R scintigraphy may be a potentially valuable tool to visualise and semiquantitate tumour GRP-R expression in a non-invasive way. As such, GRP-R scintigraphy may be helpful to 1) further elucidate the role of GRP/GRP-R in human malignancies, 2) to determine the incidence of functional GRP-R expression in various human malignancies in order to define diagnostic and staging imaging strategies and 3) to allow prediction of therapy responsiveness to recently developed bombesin antagonists.

Finally, the use of Rhenium or Indium labelled bombesin analogues may provide a new therapeutic approach to GRP/BN expressing tumours.

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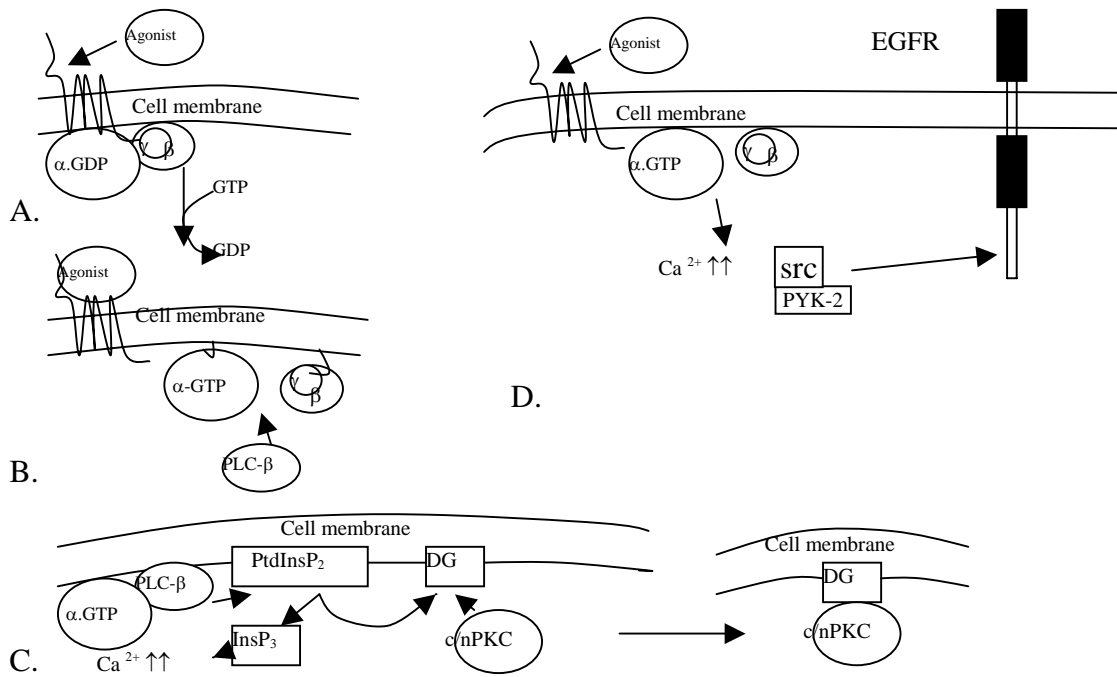
Figure 1 shows the G-protein cycle.

A. Binding of an agonist to the extracellular domain of its receptor results in the simultaneous dissociation of the heterotrimer from the receptor, exchange of GTP for GDP on the α-subunit and dissociation from the βγ-dimer.

B. Translocation of PhospolipaseC-β (PLC-β) to the cellular membrane.

C. α.GTP activates PLC-β which hydrolyses phosphatidylinositoldiphosphate (PtdInsP$_2$) to diacylglycerol (DG) and inositoltriphosphate (InsP$_3$). The hydrophobic DG remains in the plane of the membrane whereas the hydrophylic InsP$_3$ is released into the cytoplasm. The soluble cellular and nuclear Protein kinase C's (c/nPKC) bind to GD and are thus translocated to the membrane. This results in their activation with subsequent generation of cellular responses characteristic of the agonist.

D. Epidermal growth factor receptor (EGF-R) transactivation through G-protein coupled receptors can occur through activation of the protein kinases src and PYK-2, the latter are activated in the presence of increased intracellular Ca^{2+} levels.
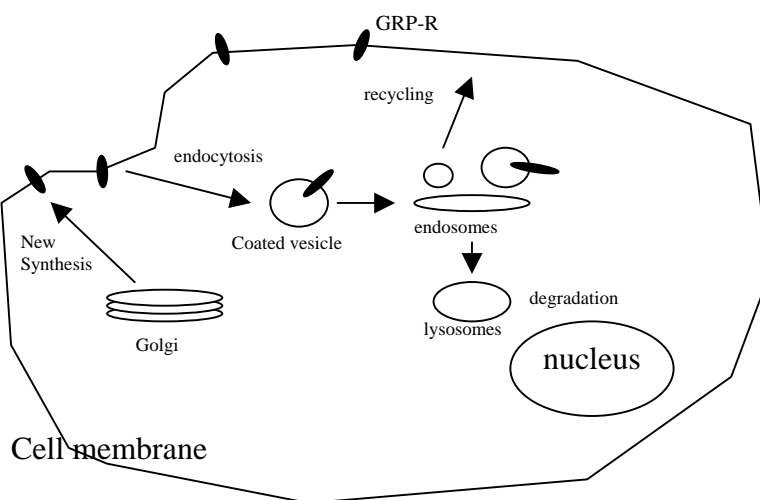
Figure 2.

Figure 2 shows a schematic representation of endocytosis and recycling of GRP-R.

Newly synthesised receptors are delivered to the cell surface from the Golgi complex. In unstimulated cells there is a relatively slow endocytosis from the surface into endosomes, which increases dramatically following binding of a receptor specific agonist. Once the receptors reach endosomes they can either be recycled back to the plasma membrane or routed to lysosomes for degradation. The ligand however remains in the perinuclear space where it is trapped in lysosomes.

BIODISTRIBUTION AND DOSIMETRY OF Tc-99m RP527, A GASTRIN RELEASING PEPTIDE AGONIST FOR THE VISUALISATION OF GRP-RECEPTOR EXPRESSING MALIGNANCIES

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ABSTRACT

Rationale : To determine the human biodistribution and radiation dosimetry of Tc-99m RP527, a promising radioligand for the visualization of gastrin releasing (GRP) receptor expressing human malignancies.

Methods : Whole body scans were performed up to 48 h after intravenous injection of 555 MBq Tc-99m RP527 in 6 subjects. Blood samples were taken at various times up to 48 h after injection. Urine was collected up to 48h after injection, allowing calculation of renal clearance and interpretation of wholebody clearance. Time-activity curves were generated for the thyroid, heart, breasts in women, testes in men and liver by fitting the organ–specific geometric mean counts, obtained from regions of interest. The MIRD formulation was applied to calculate the absorbed radiation dose for various organs.

Results : The images showed rapid hepatobiliary excretion, resulting in good imaging conditions for the thoracic region, whereas imaging of the abdomen was impeded by extensive bowel activity. Tc-99m RP527 was predominantly cleared by the kidneys and to a lesser extent by the gastrointestinal tract. At 48 h p.i. the mean excretion in the urine was 58.3 % (SD : 5.4 %). The highest absorbed doses were received by the excretory organs, i.e. the urinary bladder and gallbladder wall. The average effectve dose of Tc-99m RP527 was estimated to be 0.0095 mSv/MBq.

Conclusions : The biodistribution of Tc-99m RP527 demonstrated low lung, myocardial and liver uptake allowing early imaging of the supradiaphragmatic region and this with a dosimetry favourable for clinical SPET imaging. The amount of Tc-99m RP527 required for adequate imaging of tumoral uptake results in an acceptable effective dose to the patient.

Keywords : Tc-99m RP527 – biodistribution - dosimetry

INTRODUCTION

Gastrin releasing peptide (GRP) belongs to the family of bombesin-like peptides (BLP's) that includes the amphibian peptide bombesin as well as the mammalian counterpart GRP and neuromedin B (1). Aside from their physiological role e.g. secretion of gastrin and pancreatic enzymes and gallbladder contraction (2,3), BLP's have been shown to play a role in various tumor models and human cancer.

GRP was first shown to be a mitogen for Swiss 3T3 murine embryonal fibroblasts and subsequently a growth factor for a number of normal cell types and cancers cell lines grown in culture or as xenografts in nude mice (4,5,6,7). BLP's have been implicated as autocrine factors promoting growth of small cell lung cancer (1). Bologna et al. showed that GRP can stimulate proliferation of the androgen independent human prostatic carcinoma cell line PC3 (8). Nelson et al. found that both bombesin and GRP stimulate proliferation of breast cancer cell lines when culture serum was stripped of

endogenous GRP-like peptides (9); other workers confirmed these findings (10).

Importantly, the tumour growth stimulating effect of BLP's can be blocked by GRP antagonists. Pinski et al. (11), Milovanovic et al. (12) and Jungwirth et al. (13) demonstrated the inhibitory growth effect of antagonists of the GRP receptors in prostatic carcinoma. The same observation was reported by Koppan et al. (14) and Miyazaki et al. (15) in small cell lung- and breast carcinoma models respectively, either in cancer cells in vitro or xenografts grown in syngeneic rats or nude mice.

GRP and GRP antagonists mediate their action through membrane-bound receptors. Three structurally and pharmacologically distinct bombesin receptor subtypes have been isolated and characterized : the gastrin releasing peptide receptor (GRP-R), the neuromedin B receptor (NMB-R) and bombesin receptor subtype-3 (BRS-3). The three receptors are structurally related, sharing about 50 % amino acid identity, and are members of the G-protein coupled receptor superfamily with a seven predicted transmembrane segment topology (16). GRP receptors have been detected in various types of human carcinoma. Markwalder et al. described massive GRP receptor overexpression in human prostatic tissues that are neoplastically transformed or, like prostatic intraepithelial neoplasias, are in the process of malignant transformation (17). Gugger et al. using in vitro autoradiography showed heterogeneous GRP receptor specific expression in 43 out of 60 (71%) breast carcinomas with all involved lymph nodes from primary carcinomas expressing GRP receptors being positive (18). Corjay et al. confirmed the presence of functional GRP receptors in human lung carcinoma (19). The presence or absence of receptors as well as their level of expression has been correlated with cancer prognosis in several settings. The hormone-responsiveness and improved prognosis related to estrogen and progesteron receptor (ER and PR) expression in breast cancer is well known (20). In laryngeal cancer (21) as well as non small cell lung cancer patients (22), the expression of epidermal growth factor correlated with a worse prognosis. A similar role for GRP receptor expression as independent prognosticator was recently suggested by Toi-Scott et al in lung carcinoma patients (23). In their series, patients from whom cell lines expressed GRP receptors versus those whose cell lines did not showed a trend towards worse survival (211 $\pm$ 114 days vs. 367$\pm$ 274 days). A prognostic role for GRP was further suggested by Saurin et al. who showed that in human colon carcinoma high GRP receptor mRNA levels are related to tumour dedifferentiation and lymphatic vessel invasion (24).

In analogy to the diagnosis of somatostatin receptor-expressing tumors in vivo with somatostatin receptor scintigraphy, in vivo GRP receptor scintigraphy may be a potentially valuable tool to visualize and semiquantitate tumoral GRP expression in a noninvasive way. This may allow prediction of therapy responsiveness to bombesin antagonists and in vivo prognosis stratification. Tc-99m RP527, a targeting peptide derived from bombesin linked at its N-terminus via a linker group to a peptide sequence which chelates Tc-99m, binds GRP receptors with similar affinity to bombesin and is taken up by prostate-, pancreas- and breast tumour tissue in animal models as well as in humans in vivo (25, 26). This paper reports on its biodistribution and dosimetry in humans.

MATERIALS AND METHODS

Radiopharmaceutical synthesis

Tc-99m RP527 labelling was performed using a kit-formulation. 0.1 mL stannous chloride 2 mM, 0.1 mL sodium gluconate 60 mM, 1850-2035 MBq 99mTcO$_4$ in 0.3 mL 0.9 % sodium chloride and 0.5 mL sodium chloride were added to 100 µg of RP527. After 35 minutes in a boiling water bath, the reaction mixture was allowed to cool down to room temperature and injected on a HPLC system using an ethanol: water: acetic acid gradient. The radiolabelled peptide was collected at 45 min and the collected eluent was diluted with 10 mL 0.9% sodium chloride. The overall yield of the radiosynthesis was about 30 % with a radiochemical purity $\geq$ 90 % and a specific activity $\geq$ 4.32 TBq/µmol.

Subjects

This study was approved by the Medical Ethics Committee of the University Hospital and performed according to Good Clinical Practice. Six individuals, mean age : 46 yrs, range : 24-73 yrs, were included in the study. There were 3 men and 3 women. All subjects gave their written informed consent for participation in the study. All volunteers were free from illness on the basis of screening by medical history, physical examination, serum chemical analysis, complete blood cell count and urine analysis. For safety measurements, all chemical analysis were repeated during the experiment and compared to the baseline (screening) scores.

Imaging

Subjects were positioned supine with their arms alongside their body. Whole body images were

performed using a triple headed gamma camera (Irix, Picker, USA), equipped with low-energy high-resolution parallel–hole collimators. The energy peak was centered at 140 keV with a 15 % window. Whole body planar images were acquired 30 min, 1 , 3, 6, 24 and 48 h post injection. Acquisition was performed simultaneously in anterior and posterior position with a scan speed of 11.44 cm/min. Matrix size was 256x1024 pixels.

Urine sampling

For all subjects, all voided urine from time of injection until 48 h following injection was collected. The subjects were requested to collect urine prior to each emission scan and at home ad libitum. For each voidance, the urine was collected in a separate container and the volume and time of voidance recorded. For each voidance time, two 1 mL urine aliquots were sampled and radioactivity was counted in a NaI (Tl) counter (Cobra Packards Instruments Company) after the counting efficiency of the system had been determined. The amount of radioactivity in the urine at each voidance time was expressed as percentage of the injected activity (% IA) of Tc-99m RP527.

Blood sampling

Blood samples were taken at 15sec, 30sec, 45sec, 1min, 1min15sec,1min30sec, 2min, 5min, 7.5min, 10min, 12min, 15min, 20min, 30min, 55min, 2h, 4h, 6h, 24h and 48 h following injection of the tracer. From each blood sample duplicate 1 mL aliquots were assayed for Tc-99m radioactivity as described above. Total blood volume and consequently activity was calculated using a total blood volume based on body weight and height (27) and expressed as a percentage of the injected activity (% IA).

Dosimetry

Image analysis

For quantification of radioactivity uptake after injection of Tc-99m RP527, regions of interest (ROI's) over the total body and organs of interest were drawn on the earliest images and the shapes and sizes, i.e. number of pixels, were kept constant over all subsequent images. Correction for non-specific uptake was performed by using a region over the shoulder. For each ROI, i.e. each organ, the geometric mean, corrected for physical decay, of total anterior and posterior counts was calculated. The total body geometric mean activity, calculated

on the first image (1 h p.i.) was taken as total injected activity, considering that no urine was excreted prior to the first whole body scan. The activity in the total body and different organs was expressed as the percentage of the injected activity (% IA) calculated by the following equation: (geometric mean counts in organ or total body)/(geometric mean counts in first total body) x 100.

Dosimetric calculations

For each individual, time activity curves were generated for the thyroid, heart, breasts in women, testes in men, liver and whole body. Using in house written curve-fitting software, time activity curves were generated for these organs by bi-exponential fits. Two routes of excretion were considered, i.e. urinary and faecal excretion. The faecal excretion was predicted using data from the source organs, the urinary excretion and the material excreted by the liver. Of the material excreted by the liver, 30 % was assumed to flow directly in the small intestine. Activity in the small intestine was assumed to follow the gastrointestinal kinetics as adopted in the ICRP 30 report (28). Activity in the intestines was assumed to pass through the various segments at standard rates, the mean transit time being 4 h for the small intestine, 13 h for the upper large intestine, and 24 h for the lower large intestine. The transfer rate coefficient from the gallbladder to the small intestine was taken to be 1.8 h-1, derived by taking the mean of average emptying rates reported by others.
Source organ residence times were determined from integration of the time activity curves. Residence times were then used to determine target organ radiation doses using the MIRD methodology (29) for the normal adult (30) applying the MIRDOSE software package (31).

RESULTS

After injection of approximately 555 MBq Tc-99m RP527 (maximum 3 ng/kg per subject), no adverse or subjective effects were noticed in any of the subjects. Their vital signs remained stable throughout the experiment. Moreover, no meaningful changes were observed in any of the clinical laboratory assays performed on the blood and urine specimens obtained at 1 h, 6 h , 24 h and 48 h following administration of the radioligand when compared to baseline findings. Whole body images of a male subject showing the biodistribution of radio-activity upon injection of Tc-99m RP527 at different time points post-injection are presented in figure 1. The whole body images obtained between

30 min and 24 h p.i. show most of the activity distributed in the bladder, liver, gallbladder and intestines, reflecting the known urinary and hepatobiliary excretion of peptides. Although the kidneys were not visualized in all subjects, there was activity in the urinary bladder in all subjects, indicating prompt excretion through the renal system. Uptake in the brain, myocardium and lungs was low. There was a diffuse uptake and retention of radioactivity in the normal breast tissue in females as well as in the testes in men. Although of low quality due to low counting statistics, images at 24 h as well as 48 p.i. showed most of the remaining activity distributed in the intestines. Averaged ROI data over all subjects, expressed as % IA, of the biodistribution of 99mTc-RP 527 in the total body and in various organs at the different points in time are presented in figure 2. Clearance of radioactivity from blood fitted a bi-exponential curve. The assays of blood samples showed that the elimination of activity from the blood was initially fast, such that by 20 min post injection less than 20 % of the injected dose remained in the blood . The mean calculated half-life was 1.5 min (first exponent).

The average excretion fractions predicted for faeces and measured for urine are given in table 1. Most of the administered activity was eliminated by the renal system. The mean cumulative total measured urinary excretion at 48 h post injection was 58.3 % (SD : 5.4 %) of the administered activity, whereas the mean predicted percentage of the administered activity excreted in the faeces was 3.9 % (SD : 0.8 %) at 48 h post injection.

The residence time was highest for the remainder of the body in all subjects, followed by the urinary bladder and the liver (table 2).

The mean radiation dose estimates ($\pm$ standard deviation) were calculated for each subject independently, and then averaged (table 3). The organs receiving the highest absorbed doses were involved in the excretion of Tc-99m RP527 from the body. On average, the highest dose was received by the urinary bladder (mean : 9.6^{E-2} mGy/MBq ; SD 1.15^{E-2} mGy/MBq) followed by the gallbladder wall (mean 1.69^{E-2} mGy/MBq ; SD : 0.4 mGy/MBq) and the testes (mean 1.22^{E-2} mGy/MBq ; SD : 0.3^{E-02} mGy/MBq). The estimated mean effective dose for the adult subject was 9.5^{E-03} mSv/MBq (SD : 1.1^{E-03} mSv/MBq).

DISCUSSION

The results of the present study demonstrate the favourable biodistribution of Tc-99m RP527 in humans. Moreover the results of this study demonstrate that Tc-99m RP527 is a pharmacologically safe radioligand since it did not produce any subjective or objective pharmacological effects. The lack of a pharmacological effect was expected since the dose injected ($\pm$ 3ng/kg) is only half of the dose reported of unlabeled bombesin inducing side-effects e.g. nausea, vomiting (32).

In humans Tc-99m RP527 was cleared rapidly from circulation, such that by 20 min post injection, < 20 % of the injected dose remained in the blood. The mean calculated blood clearance half-life of 1.5 min of Tc-99m RP527 is comparable to the 2.2 min of unlabeled bombesin reported in men (33). Low brain, lung and myocardial uptake and rapid hepatobiliary excretion of Tc-99m RP527 resulted in excellent imaging conditions for the supradiaphragmatic region, even at early time points (30 min) p.i. On the other hand, in addition to a predominant urinary clearance, Tc-99m RP527 was also cleared enterohepatically resulting in a high accumulation of radioactivity affecting interpretation of the abdominal region.

Of interest is the visualization of the breasts in women and testes in men.

The visualization of the breasts is in keeping with the known bombesin-like immunoreactivity demonstrated in mammary glands of various species (34,35) indicating that bombesin/GRP similar to ER and PR may be involved in the regulation of mammary cell proliferation and differentiation. Data by Halmos et al. showing a highly positive correlation (r = 0.671, p< 0.005) between the binding capacity of high affinity (Tyr 4)-bombesin binding sites and ER levels and between the concentration of low affinity (Tyr4)-bombesin binding sites and PR levels (r =0.541, p < 0.005) in biopsies of human breast carcinoma suggest a possible interaction between both receptor systems (36) .

In primates a bombesin-like peptide resulting from alternate splicing of the GRP gene has been detected in testis. In vitro, GRP at the concentration of 100 nM, added after ionophore treatment, enhanced significantly (p<0.05) human sperm motility, capacitation, zona binding and acrosome reaction thus supporting a role for BLP's in the regulation of sperm cell proliferation and differentiation (37). In this regard the visualisation of the testes in all 3 male subjects following injection of Tc-99m RP527 suggests the presence of a currently unidentified human testicular BLP receptor. Since in mouse testis the NMB-R gene expression is prominent and in rat testis the BRS-3 receptor has been shown to be expressed, it is possible that different subtypes of bombesin receptors mediate the same response in different species (38).

The MIRDOSE software provides a calculation of the effective dose as defined in the ICRP 60. Based on the mean effective dose of 9.5^{E-03} mSv/MBq obtained in this study, both patients and volunteers could easily be investigated with 555 MBq Tc-99m RP527 allowing both planar and SPECT imaging. The corresponding effective dose of 5.27 mSv is equal to the reported average effective dose per patient from nuclear medicine procedures in Europe (39) and only half of the 10 mSv upper limit average effective dose of Category II a of the World Health Organisation and Category II b of the ICRP report (40). Since the highest dose is received by the bladder wall, frequent voiding will reduce the absorbed dose to the urinary bladder. Given the 1 % probability for severe hereditary disorders in the offspring per Sv received by the testis, the associated risk related to injection of 555 MBq Tc-99m RP527 is less than 1/10000. This is low when compared to the 1.6 % prevalence of naturally occurring genetic disorders (ICRP 60).

CONCLUSION : The biodistribution of Tc-99m RP527 demonstrated low lung, myocardial and liver uptake allowing early imaging of the supradiaphragmatic region and this with a dosimetry favourable for clinical SPET imaging.

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TABLE 1. Excretion fractions (% IA) for Tc-99m RP527

Compartment	Time p.i. (h)	AVG	SD
Urine	48-measured	58.3	5.4
Faeces	48-predicted	3.9	0.8

AVG, average ; SD, standard deviation ; % IA, percentage of the activity injected

TABLE 2. Residence times (h) for Tc-99m RP527 for each source organ

Organ	AVG	SD
Gall bladder	0.106	0.031
Lower large intestine	0.031	0.009
Small intestine	0.048	0.015
Upper large intestine	0.063	0.019
Heart wall	0.025	0.004
Liver	0.264	0.053
Thyroid	0.012	0.007
Bladder	1.801	0.206
Remainder	2.325	0.197
Breasts*	0.009	0.007
Testes*	0.028	0.007

AVG, average ; SD, standard deviation, * number of subjects = 3, respectively women and men

TABLE 3. Radiation absorbed dose estimates (mGy/MBq) for Tc-99m RP527

Target Organs	AVG	SD
Adrenals	3.27^{E}-03	3.55^{E}-04
Brain	1.23^{E}-03	1.84^{E}-04
Gallbladder wall	1.69^{E}-02	4.32^{E}-03
Lower large intestinal wall	7.27^{E}-03	8.68^{E}-04
Small intestine	4.53^{E}-03	5.98^{E}-04
Stomach	2.12^{E}-03	3.20^{E}-04
Upper large intestinal wall	5.46^{E}-03	9.65^{E}-04
Heart wall	2.64^{E}-03	5.75^{E}-04
Kidneys	2.17^{E}-03	3.04^{E}-04
Liver	4.79^{E}-03	1.13^{E}-03
Lungs	1.71^{E}-03	3.23^{E}-04
Muscle	2.40^{E}-03	2.73^{E}-04
Pancreas	2.57^{E}-03	3.86^{E}-04
Red marrow	2.18^{E}-03	2.68^{E}-04
Bone surfaces	3.46^{E}-03	4.07^{E}-04
Skin	1.25^{E}-03	1.47^{E}-04
Spleen	1.80^{E}-03	2.74^{E}-04
Thymus	1.56^{E}-03	2.49^{E}-04
Thyroid	8.07^{E}-03	3.90^{E}-03
Urinary bladder wall	9.63^{E}-02	1.16^{E}-02
Uterus †	1.08^{E}-02	1.15^{E}-03
Testes †	1.23^{E}-02	2.70^{E}-03
Ovaries †	6.75^{E}-03	5.17^{E}-04
Breasts †	9.59^{E}-04	2.28^{E}-04
Total Body	2.54^{E}-03	3.17^{E}-04
Effective dose equivalent *	1.13^{E}-02	1.12^{E}-03
Effective dose *	9.53^{E}-03	1.06^{E}-03

AVG, average ; SD, standard deviation
* Units of the effective dose equivalent and of the effective dose are mSv/MBq
† gender specific AVG and SD of 3 subjects
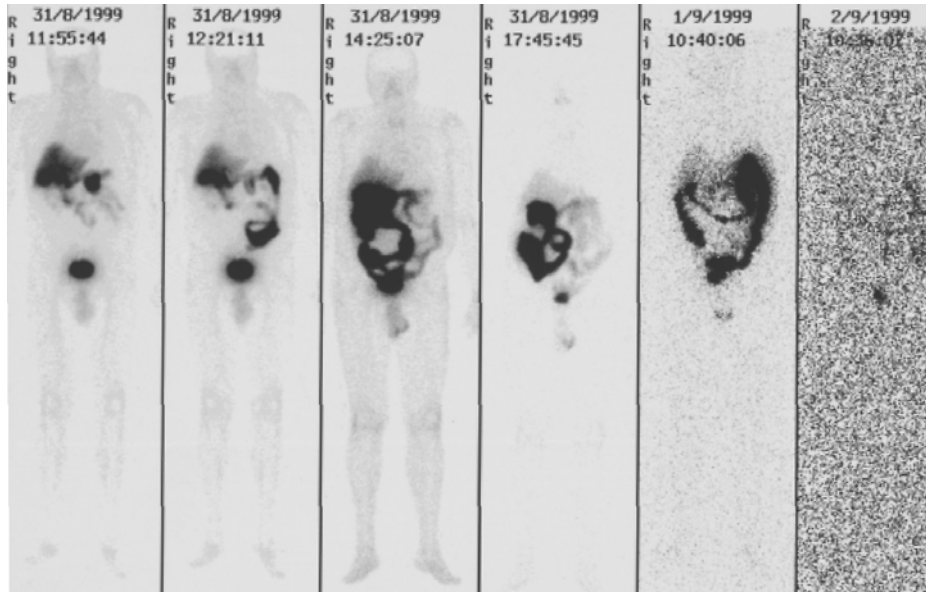
Figure 1.

The anterior whole body images obtained from a man at 30 min, 1, 3, 6 and 24 h post injection (right to left).
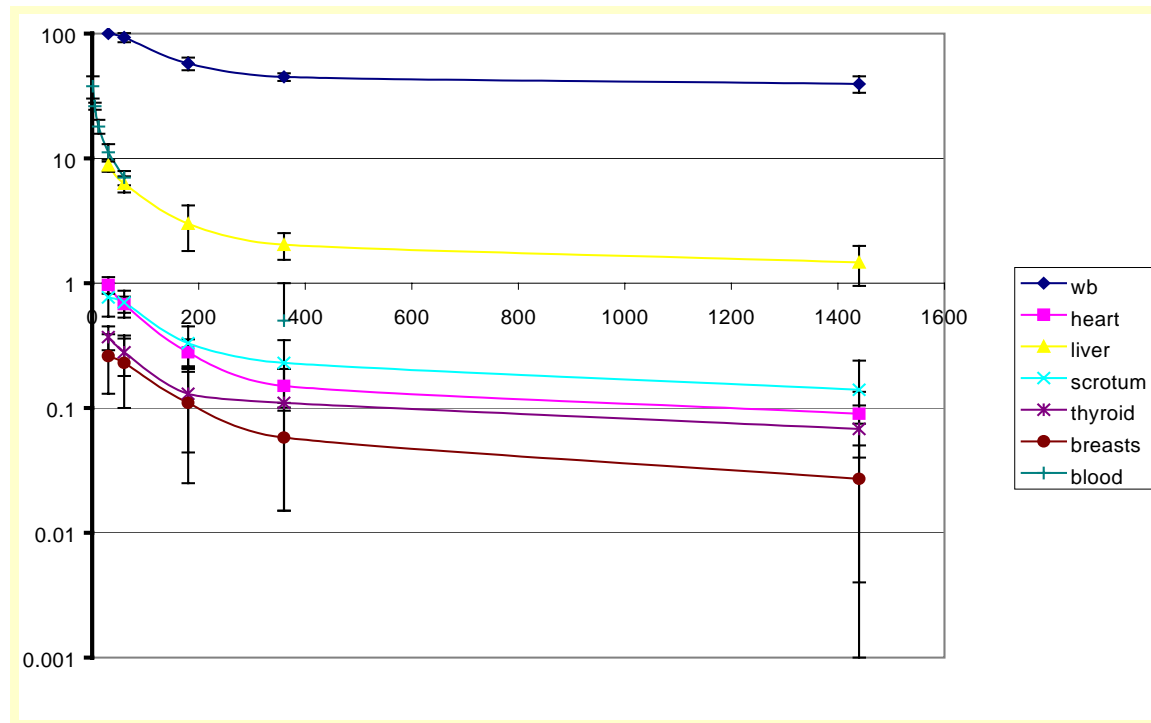
Units of the abcis and ordinate are time (h) and % injected activity respectively
Figure 2.

Time-activity curves for the total body (wb), various organs and blood, calculated from direct measurements of counts in organ-specific ROI's or in blood. The physical decay-corrected data, expressed as % IA are averaged over the 6 subjects, except for the breasts (n = 3) and testes (n=3). The error bars represent 1 SD.

Tc-99m RP527, A GRP ANALOGUE FOR VISUALISATION OF GRP RECEPTOR EXPRESSING MALIGNANCIES : A FEASIBILITY STUDY

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ABSTRACT

Gastrin releasing peptide (GRP) receptor scintigraphy could allow prediction of response to GRP receptor targeted treatment options, early non-invasive diagnosis and in vivo prognosis stratification of GRP receptor positive tumours.

This study reports on the imaging characteristics and efficacy for tumour detection of Tc-99m RP527 a Tc-99m chelated targeting peptide derived from bombesin which binds GRP receptors with high affinity.

Ten patients (4 men and 6 women, mean age : 56.4 years) either suffering from metastasised prostate- (n, number of patients = 4) or breast carcinoma (n=1) or presenting with a clinical diagnosis highly suggestive for breast carcinoma (n=5) were included in the study. In the latter 5 patients Tc-99mRP527 scintigraphy was performed prior to diagnostic eg. biopsy and staging examinations. Final diagnosis in these patients was breast carcinoma in all 5. In all patients whole body planar scans and tomographic images were acquired 1 h and 5-6 h post-injection of 555 MBq Tc-99m RP527 and tumour to normal tissue ratios (T/N) determined.

Tc-99m RP527 showed specific uptake in 4/6 breast- and 1/4 prostate carcinomas. T/N derived from planar and tomographic images increased significantly (p<0.01) from 1.65 (SD : 1.53) and 3.35(SD:3.04) to 2.58(SD:1.26) and 7.23(SD:8.46) respectively. T/N derived from tomographic images were consistently higher (p<0.01).

The data presented suggest Tc-99m RP527 results in specific tumour localisation and exhibits good imaging characteristics with good T/N that may be further enhanced by SPECT.

Key words : Tc-99m RP527- GRP receptor-feasibility

INTRODUCTION

Gastrin releasing peptide (GRP) belongs to the family of bombesin like peptides (BLP's) which include amongst others the amphibian peptide bombesin as well as its mammalian counterpart GRP (1). Aside from its physiological role (2,3), GRP has been shown to play a role in various tumour models and human carcinoma. GRP was first shown to be a mitogen for Swiss 3T3 murine embryonal fibroblasts and subsequently a tumour growth factor for a number of normal- and cancer cell lines grown in culture or as xenografts in nude mice (4,5,6,7,8). In these tumour models, the tumour growth stimulating effect of GRP is a direct result of binding of GRP to membrane G-protein coupled GRP receptors (GRP-R) on the cell surface. The mechanism by which tumour growth stimulation occurs does not appear to be constant but in general seems to involve trans-activation and up-regulation of epidermal growth factor receptors (9,10). Importantly, the tumour grwoth stimulating effect of GRP can be blocked by a wide variety of GRP antagonists ranging from synthetic octapeptides, reduced peptide bond analogues of GRP, melphalan derivatives, homodimeric forms of bombesin, the more promising substance P derivatives to anti-bombesin monoclonal antibodies eg. 2A11 (11-19). The latter has been used to treat small cell lung carcinoma patients (SCLC) and this with promising results (19). Over-expression of GRP-R when compared to normal surrounding tissue has been described in a wide variety of human tumours e.g. SCLC, prostate-, breast-, colorectal and gastric cancer (20-24). In these patient populations, treatment with GRP-R antagonists either in mono- or combination therapy, radiotherapy with radiolabeled GRP analogues and GRP-R targeted cytotoxic peptide conjugates may

prove to be new and efficient therapeutic approaches.

In analogy to the diagnosis of somatostatin receptor-expressing tumours in vivo with somatostatin receptor scintigraphy, in vivo GRP-R scintigraphy could allow prediction of response to these GRP-R targeted treatment options and early non-invasive tumour diagnosis.

This paper reports on a pilot study approved by the Ethical Board of the University Hospital Ghent, assessing the safety, imaging characteristics and efficacy for tumour detection of the GRP analogue Tc-99m RP527 developed for GRP-R scintigraphy .

METHODS AND PATIENTS

RP 527 synthesis, identification and characterisation

The peptide RP527 is synthesised chemically using solid phase tBoc chemistry and then purified by reverse-phase HPLC at Polypeptide Laboratories GmB, Wolfenbuttel, Germany. The peptide is > 97 % pure, as measured by two differential analytical HPLC methods at Polypeptide Laboratories, CA, USA. RP527 contains a tripeptide N_3S chelator (dmgly-L-ser-L-cys(acm)) which forms a stable complex with Tc-99m(V)O, losing an acetamidomethyl protecting group in the process. The chelator sequence is linked to the N-terminus of an octapeptide, Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2 derived from bombesin, via a gly-5aVa linker. The C-terminus is amidated (figure 1). The interaction of the chelator with Tc-99m and Re, a non-radioactive metal with very similar co-ordination properties, has been confirmed by NMR and X-ray crystallography (25).

Radiolabelling

Tc-99m RP527 labelling was performed using a kit-formulation provided by Resolution Pharmaceuticals and reconstituted according to the manufacturer's guidelines. 0.1 ml stannous chloride 2 mM, 0.1 ml sodium gluconate 60 mM, 1850-2035 MBq Tc-99mO_4 in 0.3 ml 0.9% sodium chloride and 0.5 ml sodium chloride were added to 100 μg of RP527. After 35 minutes in a boiling water bath, the reaction mixture was allowed to cool down to room temperature and injected on a HPLC system using an ethanol: water: acetic acid gradient in order to separate radiolabelled from unlabelled peptide. The radiolabelled peptide was collected at 45 min and the collected eluent was diluted with 10 mL 0.9% sodium chloride. The overall yield of the radiosynthesis was about 30% with radiochemical purity $\geq$ 90 % and a specific activity $\geq$ 4.32 TBq/μmol. The total peptide content of the final preparation is < 200 ng. The purified Tc-99m RP527 may be stored at 4°C in the dilution vial for up to 2 hours.

For quality control, Re chelated RP527 (RP720) synthesised by an alternative synthetic route is co-injected with the collected Tc-99m RP527 on HPLC. Both co-elute, thus confirming the identity of the complex.

Patients

Ten patients were included in the study following written informed consent.

Five patients suffered from histologically proven and documented bone metastasised androgen resistant prostate- (n, number of patients =4) or hormonal refractory bone metastasised breast carcinoma (n=1). In the latter patient, the primary tumour as well as involved lymph nodes were also present.

Another five patients were referred because of a clinical diagnosis highly suggestive for breast carcinoma (n=5). These patients underwent Tc-99m RP527 scintigraphy on the day before biopsy and/or fine needle biopsy of the suspected mass and/or lymph nodes. Also, in these patients routine staging examinations were performed within one week following Tc-99m RP527 scintigraphy.

For safety measurements, vital signs were registered and serum chemical analysis, complete blood cell count and urine analysis performed prior to injection of 555 MBq Tc-99m RP527 and repeated 1, 6 and 24 h pi.

Whole body scans were performed 1 h and 5-6 h pi. using a triple headed gamma camera (Irix, Picker) in scanning mode (11.3 cm/min) equipped with high resolution collimators. Localised SPECT acquisitions made of known tumour regions at 1 and 5-6 h with a 120 x 20 sec acquisition over 360° were reconstructed iteratively (Ordered Subset Expectation Maximisation algorithm, 2 iterations, 6 subsets) and postfiltered using a Butterworth filter (cut-off frequency : 0.8 cycles/cm, order 8). Tumour to normal tissue ratios (T/N ratio) were determined on 1 h and 5-6 h planar and summed tomographic slices incorporating the entire dimensions of the tumour.

For comparison of means, paired and unpaired two-sided Wilcoxson tests were used.

RESULTS

Patient characteristics and results are shown in tables 1 and 2.

None of the patients suffered from adverse or subjective side effects. Vital signs and clinical laboratory blood or urine assays remained stable throughout the experiment.

At 1 h. pi. the distribution of Tc-99m RP527 activity shows diffuse distribution, minimal bloodpool activity and hepatic and renal clearance. 5-6 h pi. there is significant clearance of activity with continued hepatobiliary and renal excretion. In general, breast tissue in women and testes in men showed a diffuse low tracer uptake and retention. If the pancreas was in the field of view of the tomographic images pancreatic Tc-99m RP 527 uptake was clearly depictable (6/10 patients). This was not so for planar images due to overlap with intestinal activity.

Out of four bone metastasised androgen resistant prostate adenocarcinoma patients, only one showed Tc-99m RP527 uptake in $\pm$ half of the bone lesions depicted by bone scintigraphy. In the known bone metastasised breast carcinoma patient, neither primary tumour site, involved lymph nodes nor bone metastases depicted on routine staging examinations or confirmed by histology, showed Tc-99m RP527 uptake.

All 5 patients with suspected breast lesions were tumour positive on histology. Primary tumour Tc-99m RP527 uptake was clearly depictable in 4 of them (figures 2 and 3). In these 4 patients, all involved lymph nodes and part of distant metastases (limited to the bone), when present, also showed Tc-99m RP527 uptake. No uptake was seen in areas of central tumour necrosis if present e.g. patients 6 and 9. In Tc-99m RP527 positive patients, T/N ratios derived from planar and tomographic images increased significantly from 1.65 (SD : 1.53) and 3.35 (SD : 3.04) to 2.58 (SD : 1.26) and 7.23 (SD : 8.46) at 1 h and 5-6 h pi. (p <0.01 for both planar and tomographic T/N ratios), due to a decrease in background activity. SPECT imaging showed consistently higher T/N ratios than planar imaging (p<0.001).

DISCUSSION

The primary actions of RP527 have been previously investigated in vitro and in vivo in SCID mice. Both unlabelled bombesin, uncomplexed RP527 and the structural mimic of Tc-99m RP527, RP720 (ReO-RP527) inhibited binding of I-125 bombesin to GRP-R expressing PC-3 and CF-PAC-1 cells (human prostate and pancreatic cancer cell lines respectively) with similar mean IC_{50} values ranging from 1.6-5.5 nM, suggesting similar affinities for the GRP-R. In this in vitro study, the Kd value of I-125 bombesin was 2.5 nM. Tc-99m RP527 was rapidly and specifically bound and internalised by both PC-3 and CF-PAC-1 cells. As internalisation of G-protein coupled receptor ligands following binding to its respective receptor is specific for agonists, Tc-99m RP527 is believed to be a GRP-R agonist (26). Internalisation of the GRP/GRP-R complex involves coalescence into clathrin-coated vesicles and endosomes and migration to the perinuclear space. Whereas a portion of GRP-R will recycle back to the cell membrane, the ligand remains in the perinuclear space where it is trapped in lysosomes (27,28). This entrapment prolongs the residence time of GRP agonist bound radioactivity in the target tissue when compared to antagonists, resulting in a higher accumulation of radioactivity in GRP-R positive tissues (29-30). Finally, in SCID mice studies, Tc-99m RP527 showed tumour uptake of 0.2-1.8 (μ), 0.0-0.6 (SD) %/g at 4 h with a tumour to muscle ratio ranging from 9.8-33.7 at 1 h and 6.25-25.4 at 4 h in humane prostate (PC-3), pancreatic (CF-PAC-1) and rat pancreatic (AR42J) GRP-R positive tumour cell xenografts (11).

In the study presented, Tc-99m RP527 showed selective uptake in 4/6 breast and 1/4 prostate carcinoma irrespective of lesion size. Tumour activity was more easily identified at 5-6 h pi. rather than at 1 h pi. due to a decrease in background activity, in keeping with the agonist character of Tc-99m RP527. The progressive and consistent increase in T/N ratios suggests a relative difference in clearance rate of bound and unbound radioligand from tissue and thus a specific tumour uptake mechanism in keeping with in vitro and in vivo animal data on Tc-99m RP527. A GRP-R mediated uptake is further supported by the visualisation of the pancreas, a GRP-R expressing organ, in those patients in whom the pancreas was in the field of view of the tomographic images.

The presented findings are concordant with data obtained previously using autoradiography in breast and prostate carcinoma. Gugger et al. using in vitro autoradiography showed heterogeneous GRP-R specific expression in 43 out of 60 (71%) breast carcinomas with all involved lymph nodes from primary cancers expressing GRP-R being positive (24). Low grade expression of GRP-R proved also ubiquitous in non-neoplastic human breast tissue, although heterogeneously distributed. Concordingly, Tc-99m RP527 was taken up in both primary- and lymph node lesions of 4 out of 6 (66%) breast cancer patients as well as in bone metastases if present. Low physiological uptake of Tc-99m RP527 was also seen in normal breast tissue but this did not affect tumour depiction.

Markwalder et al. (15) evaluated the expression of GRP-R in neoplastic prostate tissue and found a highly variable, high density (dpm/mg tissue: 5241$\pm$927) bombesin receptor subtype expression in 30/30 invasive prostate carcinoma. In general undifferentiated primary prostate carcinomas expressed low GRP-R levels. However poorly differentiated androgen-independent bone metastases tended to express GRP-R (4 of 7 cases) albeit in a highly variable range (dpm/mg tissue : 3863$\pm$2018). In keeping with the large standard deviation in this patient group, only one out 4 androgen independent prostate carcinomas in this series took

up Tc-99m RP527, and there appeared to be variability of uptake between different metastases, possibly reflecting different levels of receptor expression. As shown by Jiborn et al. neuroendocrine differentiation with appearance of neurosecretory granules, rich in various peptide hormones eg. GRP and biogenic amine , in prostatic carcinoma during androgen withdrawal treatment occurs in $\pm$ 40 % of untreated patients. Part of these patients might benefit from treatment with GRP antagonists e.g. MoAb 2A11 and could be selected by means of Tc-99m RP527 scintigraphy.

In conclusion, the data presented suggest Tc-99m RP527 is safe, results in specific tumour localisation and exhibits good imaging characteristics with good lesion-to-normal tissue background ratios. However, additional studies clarifying the clinical potential of this agent and correlating in vivo Tc-99m RP527 tumour uptake with the presence of the tumour GRP receptor status as measured, for example, by immunohistochemistry or radioligand binding assays are mandatory.

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Table 1

Patient	Gender	Age	Diagnosis	Planar RP527	SPECT	ΔT/N	Nb RP527+/ Nb BSL
1.	M	62	ARBMPA	-	-	na	0 /34
2.	M	63	ARBMPA	+	+	↑	14/33
3.	M	74	ARBMPA	-	-	na	0 /71
4.	M	59	ARBMPA	-	-	na	0 /38
5.*	F	62	HRBMBC	-	-	na	0 / 4

Legends: ARBMPA = androgen resistant bone metastasised prostate adenocarcinoma, HRBMBC : hormone refractory bone metastatised breast carcinoma, na = not applicable, ΔT/N = change in tumour-to-normal tissue ratio from 1 h to 5.6 h pi., + = tumour activity present, - = tumour activity not visualised, ↑ = increase, Nb RP527+ = number of RP527 positive lesions, Nb BSL = number of bone scintigraphic metastatic lesions.
* Primary tumour and axillary lymph nodes in this patient were also Tc-99m RP527 negative.

Table 1 shows the patient characteristics and details of 99mTc RP527 scan results in the subgroup of patients suffering from proven and documented bone metastasised androgen resistant prostate – or hormone ablative treatment refractory breast carcinoma.

Table 2.

Patient	Age	Final diagnosis	Planar RP-527	SPECT	ΔT/N	Nb RP527+/ Nb BSL
6.	47	T3N2M0	-	+	↑	na
7.	37	T3N1M0	-	+	↑	na
8.	50	T2N3M1	-	-	na	0/20
9.	65	T4N3M1	+	+	↑	3 /4
10.	44	T3N1M0	+	+	↑	na

Legends : na = not applicable, Δ T/N = change in tumour-to-normal tissue ratio from 1 h to 5.6 h pi., + = tumour activity present , - = tumour activity not visualised, ↑ = increase, Nb RP527+ = number of Tc-99m RP527 positive lesions, Nb BSL = number of bone scintigraphic metastatic lesions

Table 2 shows the patient characteristics and details of 99mTc RP527 scan results in the subgroup of patients referred because of a clinical diagnosis highly suggestive for breast carcinoma.

Figure 1
Figure 1 shows the structure of Tc-99m RP527.
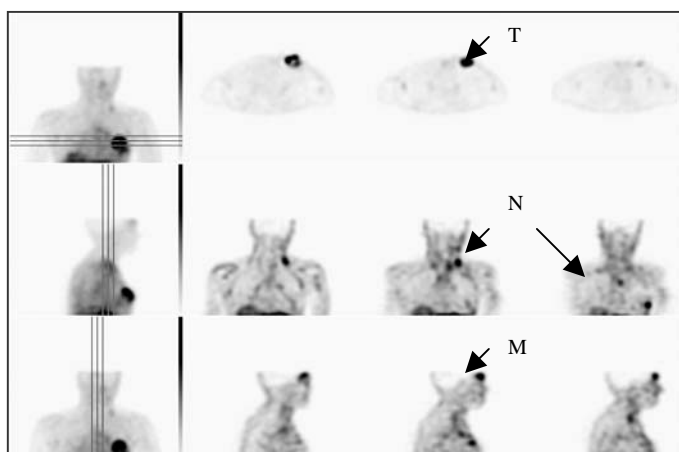
Figure 2
Figure 2 shows the whole body images (posterior and anterior projection) as well as the corresponding transaxial, coronal and sagittal tomographic (top to bottom) Tc-99m RP527 image slices of the thoracic region of patient 9 showing uptake in the primary breast tumour (indicated by T), involved axillar, retrosternal and cervical lymph nodes (indicated by N) and bone metastases (indicated by M).
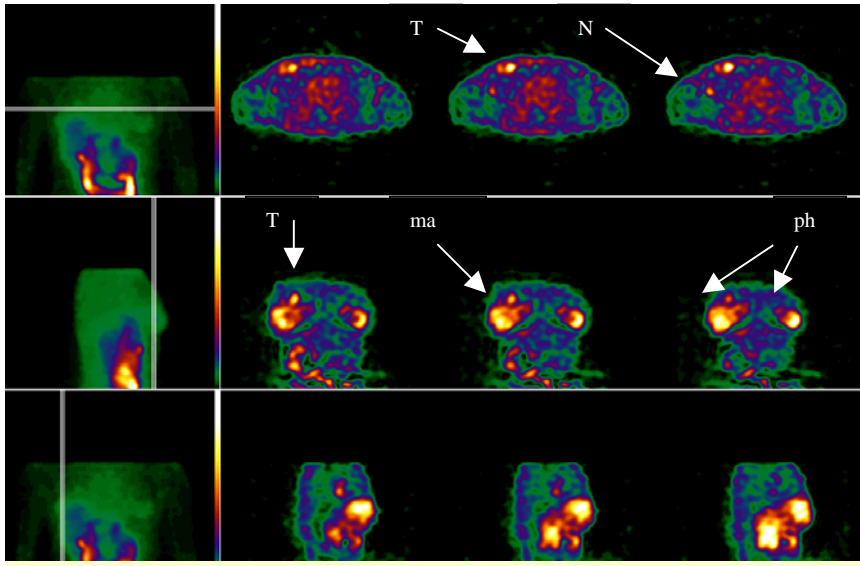
Figure 3

Figure 3 shows the transaxial, coronal and sagittal (top to bottom) tomographicTc-99m RP527 image slices of the thoracic region of patient 10 showing uptake in the primary breast tumour (indicated by T), a mastitis component (indicated by ma) and involved axillary lymph nodes (indicated by N). Bilateral, symmetrical physiological breast uptake (indicated by ph), relatively intense when compared to the other breast carcinoma patients, is also seen. Interfering abdominal activity has been removed using region growing.

GASTRIN RELEASING PEPTIDE RECEPTOR IMAGING IN HUMAN BREAST CARCINOMA USING Tc-99m RP527 VERSUS IMMUNOHISTOCHEMISTRY

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ABSTRACT

Background : In vivo visualisation of gastrin releasing peptide receptor (GRP-R) status by means of 99mTc RP527 could predict or rapidly assess respons to treatment modalities targeting GRP-R. We report on the relationship between breast tumour uptake of 99mTc RP527, a 99mTc chelated peptide which binds to GRP-R, and GRP-R expression as measured by immunohistochemistry (IHC).

Methods : Nine patients referred because of a clinical diagnosis suggestive for breast carcinoma, four patients suffering from tamoxifen resistant bone mestastasised breast carcinoma and one patient presenting with a local breast carcinoma recurrence invading the sternum underwent 99mTc RP527 scintigraphy. Results obtained were compared to routine staging examinations in all patients and to routine histopathologic analysis and IHC GRP-R staining in the first nine patients.

Findings : All 9 patients with suspected breast lesions were tumour positive on histology. Uptake of 99mTc RP527 was clearly depictable in the primary tumour of 8 out of 9 patients and in involved lymph nodes and part of the distant metastasis limited to the bone (occurring in only one patient) when present. In none of the remaining patients, 99mTc RP527 uptake was found.

In 5 out of 8 99mTc RP527 positive patients, IHC was negative whereas the one 99mTcRP527 negative tumour was also IHC negative.

Interpretation : The different incidence GRP-R number found between 99mTc RP527 imaging, and IHC likely relates to differences in methodology. In the near future, 99mTc RP527 imaging might be helpful to further elucidate the role of GRP-R in human malignancies and to allow prediction or monitoring of therapy responsiveness to recently developed GRP-R antagonists.

INTRODUCTION

The duration of remission of breast cancer produced by currently available endocrine treatment modalities is varied and eventually all patients with advanced breast cancer treated by any modality of estrogen deprivation relapse (1). Thus, there exists a need for new endocrine treatment modalities that improve outcome for hormone unresponsive breast tumours. Gastrin releasing peptide/bombesin (GRP) has been shown to function as an autocrine growth factor in a variety of human tumours, including breast carcinoma (2). Its tumour growth stimulating effect is a direct consequence of binding to the GRP heptaspanning receptor (GRP-R). With the aim of inhibiting breast tumour growth, this growth factor and its receptor have been evaluated as potential targets for various antagonists or blokking agents, e.g. the monoclonal antibody 2A11 (3,4,5,6). As these blocking agents result in tumour stasis rather than regression, alternative techniques to morphological imaging, assessing changes in tumour volume, will be necessary to monitor there efficacy.

The success of [111]In Octreoscan in demonstrating tumours with somatostatin receptors (7) and of [I123] vasoactive intestinal peptide for visualization of tumours overexpressing VIP receptors by scintigraphic imaging (8), prompted the search for a radiolabelled bombesin/gastrin releasing peptide analogue. By virtue of their potential to visualise tumour lesion GRP-R status and its effective downregulation following efficient treatment, radiolabelled gastrin releasing peptide analogues have

the potential to predict or rapidly assess respons to GRP-R targeted treatment modalities.

Tc99m RP527 consists of a targeting peptide derived from bombesin, linked at its N-terminus via a linker group to a peptide sequence which chelates 99mTc. Both unlabelled bombesin, uncomplexed RP527 and the structural mimic of ^{99m}Tc RP527, RP720 (ReO-RP527) inhibit binding of ^{125}I bombesin to GRP-R expressing PC-3 and CF-PAC-1 cells (human prostate and pancreatic cancer cell lines respectively) with similar mean IC_{50} values ranging from 1.6-5.5 nM. Additionally, ^{99m}Tc RP527 was rapidly and specifically bound and internalised by both PC-3 and CF-PAC-1 cells (9). The ligand has been shown to be safe and to exhibit good imaging characteristics (10). Data on its uptake as compared to GRP-R measurements by other techniques, however, are lacking.

This paper reports on the relationship between 99mTc RP527 uptake in tumour lesions of patients suffering from breast carcinoma and GRP-R expression as measured by immunohistochemistry (IHC).

PATIENTS AND METHODS

PATIENTS

This study was approved by the Medical Ethics Committee of the University Hospital and performed according to Good Clinical Practice. Fourteen patients, mean age : 58.6 yrs, range 37-75 yrs, were included in the study. All patients gave their written informed consent for participation in the study. Nine patients were referred because of a clinical diagnosis highly suggestive for breast carcinoma. These patients underwent Tc-99m RP 527 scintigraphy on the day before biopsy and/or fine needle biopsy of the suspected mass and/or lymph nodes. In addition to routine histopathologic analysis, slides of these patients were stained for GRP-R. Also, in these patients, routine staging examinations were performed within one week following Tc-99m RP527 scintigraphy.

Another four patients suffered from histologically proven and documented tamoxifen resistant bone metastasised breast carcinoma and one patient presented with a local recurrence invading the sternum.

METHODS

Radiopharmaceutical synthesis

99mTc-RP 527 labelling was performed using a kit-formulation. 0.1 mL stannous chloride 2

mM, 0.1 mL sodium gluconate 60 mM, 1850-2035 MBq 99mTcO4 in 0.3 mL 0.9 % sodium chloride and 0.5 mL sodium chloride were added to 100 µg of RP527. After 35 minutes in a boiling water bath, the reaction mixture was allowed to cool down to room temperature and injected on a HPLC system using an ethanol: water: acetic acid gradient. The radiolabelled peptide was collected at 45 min and the collected eluent was diluted with 10 mL 0.9% sodium chloride. The overall yield of the radiosynthesis was about 30 % with a radiochemical purity $\geq$ 90 % and a specific activity $\geq$ 4.32 TBq/µmol.

Scintigraphy

Subjects were positioned supine with their arms alongside their body. Whole body images were performed using a triple headed gamma camera (Irix, Picker, USA), equipped with low-energy high-resolution parallel–hole collimators. The energy peak was centered at 140 keV with a 15 % window. Whole body image scans were acquired 1 h and 5-6 h pi. in scanning mode (11.3 cm/min). Localised SPECT acquisitions made of known tumour regions at 1 h and 5-6 h with a 120x 20 sec acquisition over 360 ° were reconstructed iteratively (Ordered Subset Expectation Maximisation algorithm, 2 iterations, 6 subsets) and postfiltered using a Butterworth filter (cut-off frequency : 0.8 cycles/cm, order 8). Tumour to normal tissue ratios (T/N ratio) were determined on 1 h and 5-6 h planar and summed tomographic slices incorporating the entire dimensions of the tumour. The normal distribution of early and late T/N ratios was assessed using a Chi-square test. Differences between T/N ratios obtained 1 h and 5-6 h pi. were assessed using a paired t-test.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded samples held by the Department of Pathology of the University Hospital Ghent. The serie consisted of 7 diagnostically obtained specimens (6 surgical biopsies and 1 needle biopsy) and 2 curative specimens. 5 µm thick sections were cut and mounted on poly-L-lysine-coated slides. No antigen retrieval procedure was applied. GRP-R expression was evaluated using a polyclonal rabbit anti-peptide antibody (generously provided by RV Benya, University of Florida) in a standard peroxidase-based three-stage procedure (11). In all instances, antibodies were used at a 1:500 dilution, predetermined as optimal by dilution titration using small cell lung carcinoma, GRP-R expressing tissue, as a positive control. Adjacent non-malignant glands served as neagtive controls.

All specimens were evaluated using a Nikon E600 microscope with Axioplan objectives connected to a Microlumina ultraresolution scanning digital camera (3,380 x 2,700 pixels (Leaf systems, Fort Washington, PA))

To allow a more direct comparison with 99mTc RP527 tumour uptake, reflecting both number of GRP-R expressing cells and the intensity of GRP-R expression by these cells, slides were analysed for geographic extent and intensity of staining. Ten or more (10+) high-power fields (hpf) containing tumour as well as an equal number of normal mucosal fields at x400 magnification were evaluated for each slide. For geographic extent determination, fields were scored as 1+ = <25%, 2+ = 25-50%, 3+ = 50-75%, and 4+ = > 75% cells/hpf positive for chromogen. In terms of intensity, cells were scored on a three point scoring scale, score 1 = low staining intensity, score 2 = moderate staining intensity, score 3 = high staining intensity. Subsequently, a summed score defined as the geographic extent score multiplied by the corresponding intensity score was determined for each tumour. Only slides with a summed score > 4 were considered positive for GRP-R.

RESULTS

After injection of approximately 555 MBq 99mTc RP527 (maximum 3 ng/kg per subject), no adverse or subjective effects were noticed in any of the subjects. The whole body and tomographic images obtained at 1 and 5 h pi. show a diffuse and heterogeneous uptake of radioactivity in the normal breast tissue, limited to the central, peri-aureolar glandular part of the breast.

Results of the nine patients referred because of a clinical diagnosis highly suggestive for breast carcinoma are shown in table 1. All 9 patients with suspected breast lesions were tumour positive on histology. Primary tumour 99mTc RP527 uptake was clearly depictable in 8 out of 9 patients. In these 8 patients, involved lymph nodes and part of the distant metastases limited to the bone (occurring in only one patient), when present, also showed 99mTc RP527 uptake. In 99mTc RP527 positive patients, T/N ratios derived from tomographic images increased significantly from 3.8 (sd : 2.6) at 1 h p.i. to 11.3 (sd : 10.2) and 5-6 h p.i.(p = 0.0007). In contrast to normal breast epithelium, increased receptor immunostaining was observed in 3 out of 9 primary breast tumours, respectively one ductal and two lobular carcinoma. Staining was predominantly cytoplasmatic and to a lesser extent membranous and limited to the well differentiated areas of the tumours (see figure 1). All three patients showed clearly depictable 99mTc RP527 uptake. In one patient both GRP-R staining and 99mTc RP527 uptake were absent. In the 5 remaining patients, tumour GRP-R staining was negative whereas 99mTc RP527 uptake was positive.

Finally, in none of the 4 bone metastasized breast carcinoma patients, nor in the patient presenting with a local recurrence involving the sternum, osseous involvement was visualised by means of 99mTc RP527 scintigraphy.

DISCUSSION

The data presented show 99mTc RP527 uptake in untreated primary breast carcinoma and related metastases, but not in metastases of previously treated patients, as well as ubiquitous and heterogenous 99mTc RP527 uptake in non-neoplastic breast tissue.

Expression of GRP-R in primary human breast carcinoma has been previously studied by other methodologies than scintigraphy or immunohistochemistry. Using receptor binding techniques on tumour homogenates, Halmos et al. found that 33% (33/100) of primary breast carcinoma were GRP-R positive (12). Contrarywise, using in vitro autoradiography Gugger et al. reported a 62% (44/71) incidence of GRP receptors in primary breast carcinomas (13). In the series presented, 8 out of 9 (88%) primary breast carcinoma were GRP-R positive as measured by in vivo 99mTc RP527 scintigraphy whereas only 3 out of eight scintigraphy positive patients proved positive on immunohistochemistry. The single scintigraphy-negative patient was also negative on IHC.

The different incidence numbers found by these differing techniques likely relate to differences in patient inclusion and methodology. GRP receptors in primary breast carcinoma are often heterogeneously distributed. Thus surgical sections of tumour fragments obtained for IHC may contain an insufficient or unrepresentative number of tumour cells to provide a positive staining result. A similar heterogenous distribution has been reported previously in breast carcinoma for other receptors, e.g. alpha estrogen – (αER), progesterone- (PR) and somatostatin receptors (14,15). Furthermore, GRP-R are thermolabile proteins, thus sub-optimal storage and assay conditions for in vitro analysis may destroy binding activity leading to false negative IHC results. On the other hand, in terms of imaging, peptide bombesin derivatives may also bind to neuromedin-B receptors (NMB-R), albeit with much lower affinity (16). The latter is structurally related to the GRP-R

sharing about 50 % of amino acid identity (17). However, there is no evidence for NMB-R expression in breast carcinoma (13). Additionally, the blood clearance half-life of 99mTc RP527 derived from the first exponent of the bi-exponential blood clearance clearance curve obtained in healthy volunteers is 1.5 minutes (18). Consequently, binding of 99mTc RP527 to its corresponding receptors has to occur very rapidly following ligand injection which for low affinity receptors would result in a faint and probably undiscernable tumour uptake of 99mTc RP527 when compared to background activity.

Similar to the findings by Gugger et al. if the primary tumour was GRP-R positive as assessed by 99mTc RP527 scintigraphy, involved lymph nodes were also positive and easily depictable, even if a single lymph node was involved (13). This is not surprising given the high GRP receptor density and homogeneous distribution in axillary lymph node metastases compared with the lack of GRP receptors in surrounding lymphoreticular tissue (high tumour to background ratio).

In the one patient presenting with a primary breast carcinoma and osseous involvement, both primary but only half of the metastatic bone lesions took up 99mTc RP527 probably reflecting the difference in clonal origin, with distinct biological parameters, of metastatic lesions. On the other hand, in the five patients showing either tumour recurrence under adjuvant tamoxifen treatment or formerly treated for their osseous disease, none of the known sites of osseous involvement were positive on 99mTc RP527 scintigraphy. The absence of uptake in these less differentiated tumours when compared to untreated primary tumours as well as the previously reported positive association between GRP-R and αER (12) and the preferential expression on well differentiated areas of tumour as demonstrated by immunohistochemistry in this study supports the notion that apart from its known mitogenic and growth stimulating activity (2), GRP-R could also act as a morphogen or differentiation factor in breast carcinoma. A role for GRP-R as a morphogen has been previously reported in colon carcinoma (11). In this regard, GRP is known to activate multiple different intracellular signaling pathways, including those that stimulate cell-growth, cell differentiation and cell-to cell attachment (2). Depending on the cell type, the GRP-R couples to multiple different G proteins, including members of the p^{21ras} superfamily (19). GRP-R activation of these proteins, including p^{21rho}, alters p^{125fak} phosphorylation and influences the integrity of focal cell-cell adhesions (20). Because in normal development many morphogens act via heptaspanning receptors, it is not surprising that some

have recently been shown to perform this role in cancer, e.g. vasoactive intestinal peptide (21,22,23).

Finally, Gugger et al. described a ubiquitous heterogeneous GRP-R expression in normal breast ductules and lobules, possibly related to a heterogenous innervation pattern of the glands and lobules, assuming that GRP plays a neurotransmitter role in the breast, as it does in the gastro-intestinal tract (13). As the sample size containing non-neoplastic breast tissue was often small, the authors suggested that the percentage of receptor heterogeneity may not be representative for the whole breast. The 99mTc RP527 images show the heterogeneous GRP-R expression to be limited to the central, glandular part of the breast. The reason for this heterogeneity and the relative high incidence when compared to other hormone receptors e.g. αER and PR, warrants further investigation. Possibly GRP-R, similar to αER and PR may be involved in the regulation of mammary cell proliferation and differentiation.

In the direct future, traditional objective end points such as simple tumor cell killing or growth delay will no longer by themselves be sufficient for evaluating response to treatment of novel developed anti-cancer treatment modalities (24). The opportunity presented is to replace such blunt instruments with more specific end-points that are tailor-made for any particular molecular target. One of these surrogate end-points is the use of nucleic acid microassays and related proteomics on tumour biopsy tissue (25,26). However, as it will be impossible to obtain information on all lesions by these techniques, tumor heterogeneity will need to be addressed by alternative methodologies. In this regard, the development of non-invasive whole body molecular imaging approaches is extremely attractive. As companies are further reducing the time scale from potential new targets to development candidates, these imaging techniques will need to be developed simultaneously. Over the last few years, potent and selective bombesin antagonists have been developed. These compounds have shown strong inhibition in both animal tumour models and human cell lines and more recently also in patients suffering from small cell lung carcinoma (2,3,4,5). Therefore, the application of this type of compound as a long-term treatment of breast cancer may be considered as a future therapeutic goal for which the appropriate imaging technique could be 99mTC RP527 scintigraphy. As shown in the series presented, in a subset of patients, 99mTc RP527 scintigraphy allows visualization of primary breast carcinoma as well as involved lymph nodes and metastases. These patients may ultimately prove more amenable for GRP-R targeted treatments. In this regard, the presence of GRP-R was shown to correlate

with growth inhibition by an anti-bombesin antibody in small cell lung cancer cell lines (27). Additionally 99mTc RP527 scintigraphy has the potential to rapidly (sequential imaging) and non-invasively monitor effective dowregulation or blockage by GRP-R targeted treatments.

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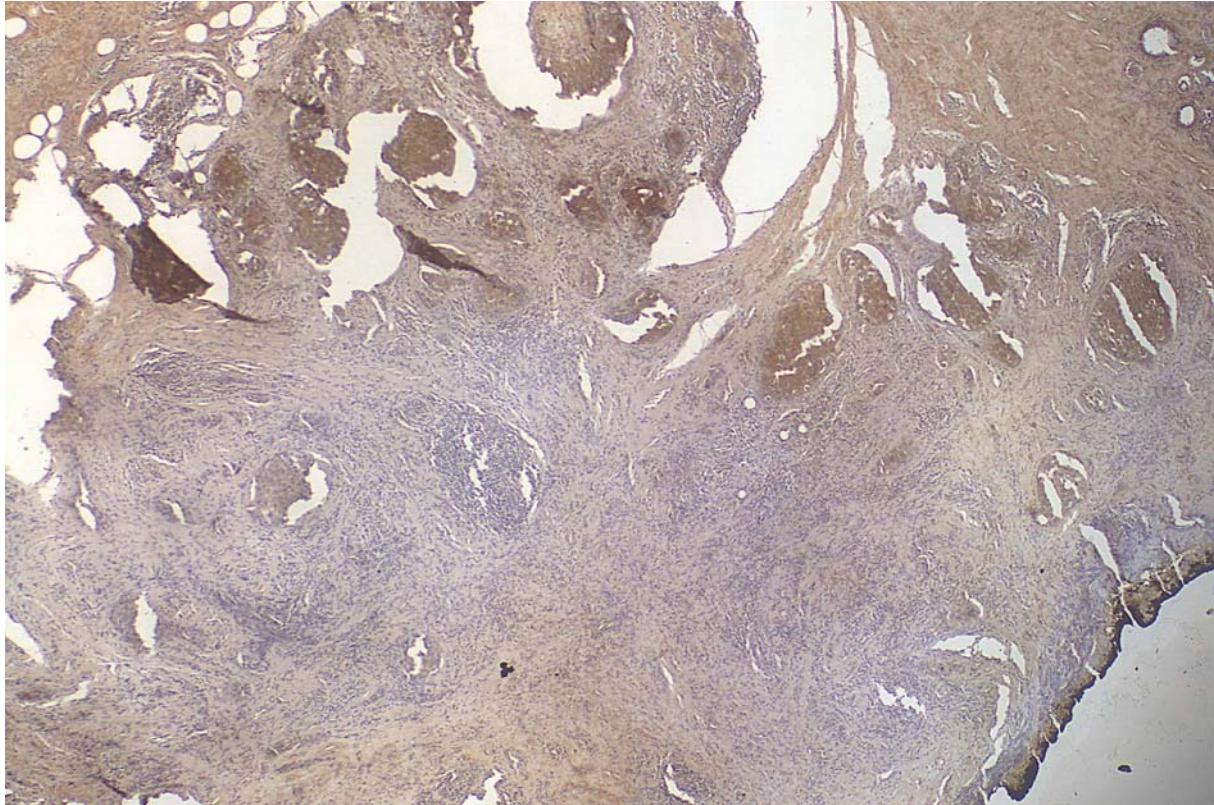
Figure 1 shows GRP-R staining, using a liquid DAB substrate-chromogen, in breast tumour areas with a more glandular structure of a 44-year-old patient presenting with an invasive ductal carcinoma. Corresponding scintigraphic images showing 99mTc RP527 uptake in the primary tumour as well as in involved lymph node tissue are displayed on page 77.

table 1. Clinical, pathological and scintigraphic findings in patients presenting with a primary untreated breast carcinoma.

Age (yrs)	Histology	TNM stage	αER IHC	PR IHC	GRP-R (SS) IHC	Imaging (T/N ratios primary , early/late)
37	Inv.Lob.	TxN1M0	0	0	negative	positive (2.1/9.5)
72	Inv.Lob.	T3N+M0	0	0	negative	positive (1.8/2.1)
63	Inv.Lob.	T1N0M0	90	90	negative	positive (4.3/12.3)
69	Inv.Duct.	T2N1M0	75	60	negative	positive (3.6/4.5)
44	Inv.Duct.	T3N1M0	0	0	positive (12)	positive (3.6/4.6)
41	Inv.Lob.	TxNxM0	90	80	positive (6)	positive (3.4/8.2)
75	Inv.Duct.	T4N1M0	95	0	negative	positive (8.5/32.7)
51	Inv.Lob.	TxNxM0	90	50	negative	negative (-/-)
65	Inv.Lob.	T4N1M1	98	5	positive(6)	positive (11.9/39)

TNM, Tumour Node Metastases stage, IHC = immunohistochemistry, αER = α estrogen receptor , PR = progesterone receptor, T/N = Tumour to normal tissue ratios obtained 1(early) and 4-5 h(late) post injection for the primary tumour, GRP-R = gastrin releasing peptide receptor.

For αER and PR, IHC results are expressed as the percentage of positive tumour cells. For GRP-R, IHC is expressed as a sum score (SS), taking into account both intensity and the number of GRP-R positive cells.

GENERAL DISCUSSION

DISCUSSION

Part I

In part I of the thesis the available literature on radiolabelled estradiol derivatives to predict response to hormonal treatment in breast cancer is reviewed and the human biodistribution, dosimetry and metabolisation of *trans*–1-(4-(2-diethylaminoethoxy)phenyl)-1,2-diphenyl-5mesyl-1-pentene (iodomethyl-*N,N*-diethyltamoxifen (ITX) in healthy volunteers as well as preliminary findings on ITX uptake in patients suffering from breast carcinoma described.

It was shown that available data fail to substantiate a direct relationship between tumour uptake of radiolabelled estradiol derivatives and αER (alpha estrogen receptor) status in keeping with recent pathophysiological findings demonstrating 1) estradiol sequestration and retention through other than αER mediated, either membrane or non-membrane related, mechanisms and 2) an inverse relationship between estradiol uptake and local biosynthesis through aromatisation and interconversion in αER positive tumours (1). Additionally, given the discovery of very high affinity αER-like binding sites (Kd, dissociation constant < 0.1 nM) (2) and the potential for underestimation of αER Kd when using ligand binding assays (3), at least part of radiolabelled estradiol derivative uptake reflects tumoral perfusion rather than the ligand-receptor binding process. However, the reduction in cellular uptake, membrane sequestration and local biosynthesis of estradiol following tamoxifen treatment in αER responsive tumours, should allow for early therapy respons prediction through rapid sequential radiolabeled estradiol scintigraphy (1). The need for two scanning episodes however may be considered a relative drawback. Consequently an imaging technique that could give the same information in only one scanning episode is of interest. In this regard, assessment of uptake of radiolabelled tamoxifen, the mainstay of adjuvant- as well as palliative hormonal treatment for breast cancer patients, seems a plausible alternative. Not only could in vivo imaging of radiolabelled tamoxifen tumour uptake, retention and eventually efflux help to increase our understanding of tamoxifen's action, it could also help to increase our knowledge of the mechanisms involved in resistance to the drug. This in turn could provide new targets for the design of therapeutic agents which can both effectively antagonise the αER-dependent growth pathway, as well as circumvent or prevent the emergence of inevitable resistance to the drug in vivo.

Yang et al. previously introduced *trans*–1-(4-(2-diethylaminoethoxy)phenyl)-1,2-diphenyl-5mesyl-1-pentene(iodmethyl-*N,N*-diethyltamoxifen, ITX) as an αER specific anti-oestrogen for breast cancer imaging (4). ITX was shown to actively reduce the growth of MCF-7 human breast cancer cells grown in nude mice, displaying similar growth inhibitory activity when compared to tamoxifen. Furthermore, in gamma scintigraphic imaging studies with ITX, the rabbit uterus uptake could be blocked by pretreatment with diethylstilbestrol, suggesting the uptake in uterus is via an αER-mediated process. In terms of biodistribution, injection of ITX in Sprague-Dawley and Fisher 344 rats showed high liver (6.764 % $\pm$ 0.056% injected dose/g tissue at 1 h) and lung (2.835% $\pm$ 0.1596% injected dose/g tissue at 1 h) uptake as well as progressively increasing tumour to blood ratios reaching an optimum at 24h p.i. These findings implied that delayed imaging may be required in order to increase the ITX tumour to tissue ratio and clear the high amounts of drug taken up by the liver and lung, allowing better depiction of ITX uptake by tumours. The human biodistribution and dosimetry study however showed low lung retention and rapid hepatobiliary excretion of the radiopharmaceutical, resulting in excellent imaging conditions for the thoracic region even at early time points (1 h) post injection (5). On the other hand, enterohepatic clearance resulted in high accumulation of radioactivity in the abdominal region, impeding image interpretation. In particular, expected physiological uptake in normal uterine tissue was difficult to discern from bowel or urinary bladder activity. Differences in clearance between rats and humans may be due to species differences in metabolism, e.g; a more rapid biliary clearance in man. Based on an effective dose of 8.44^{E}-04 (SD: 1.51^{E}-03 mSv/MBq) as calculated by the MIRDOSE software (6,7), it was shown that both patients and volunteers could easily be investigated with 185 MBq ITX, allowing both planar and single-photon emission tomography (SPECT) imaging.

As labeled metabolites not only could complicate quantitative analysis but also provide an image background that could make it difficult to identify ITX binding lesions that are small or display low ITX binding, the clearance properties and metabolite formation of ITX in human plasma were studied. ITX was rapidly cleared from human plasma according to a two-compartmental model with polar metabolites appearing as early as 7.5 min p.i., dominating by 20-90 min p.i., in keeping with the rapid liver uptake seen in the human biodistribution and dosimetry study. As shown by Jacolot et al., the metabolisation of tamoxifen occurs predominantly in the liver via cytochrome P-450 enzymes located in the microsomes (8), the major metabolite being N-demethyltamoxifen accounting for 95-100% of all tamoxifen serum metabolites (9). Although N-desmethyltamoxifen is an active metabolite, its binding affinity for the αER is much lower than the parent compound. It

has also been noted to be much less potent than tamoxifen at blocking estrogen-induced increases in uterine weight. 4-Hydroxylation is another primary metabolic pathway for tamoxifen. Although the anti-estrogen 4-hydroxytamoxifen is present in much smaller concentrations than the N-desmethyl metabolite it has a much higher binding affinity to the αER. Finally, a number of other metabolites e.g. metabolite E, formed by cleavage of the dimethylaminoethane basic side chain of tamoxifen with subsequent conversion to a hydroxyl group, and bisphenol, both eliciting estrogen activity have been reported. ITX is structurally similar to tamoxifen and the CH_3I group does not interfere with the demethylation, hydroxylation nor deamination proces. Thus, N-desmethyl-ITX as well as hydroxylated or deaminated ITX derivatives could contribute to a certain extent to human breast carcinoma visualisation through direct binding to the αER. Yet the radioactive metabolite found in man can not be N-demethyl ITX, 4-OH-ITX nor any other anticipated metabolic derivative. As N-dimethyl ITX, 4-OH-ITX or deaminated ITX derivatives are more polar than ITX, their HPLC retention time should be reduced to a certain degree when compared to ITX which was not subtantiated by our findings. Rather, the metabolite formed eluted with the deadtime of the HPLC column suggesting the presence of a very small, highly polar metabolite excluding any of the abovementionned metabolites. In this regard, the biodistribution data in humans showing visualisation of the thyroid over time point towards free iodine. As iodine is polar and negatively charged, with the exception of the thyroid, it has only limited access to the intracellular space. However, it will increase background activity owing to its presence in blood and extracellular space. Given the early blood disappearance of ITX and the slow decline of metabolites, from the standpoint of imaging and quantifying ITX tumour uptake, it could be concluded that delayed imaging is unlikely to yield better results than imaging starting at 15-30 min p.i.

The feasibility of ITX imaging was assessed in patients suffering from primary- and known metastatic breast carcinoma, either resistant or non-resistant to hormonal treatment. In previously untreated patients, ITX uptake was related to tumor αER and PR receptor status as assessed by immunohistochemistry. In primary breast carcinoma patients, increased uptake when compared to normal anticipated physiological αER expressing breast uptake was seen in αER+ve/PR +ve tumours but not in αER+ve/PR-ve and αER-ve/PR-ve tumours. As shown in the Early Breast cancer Trialists meta-analysis, whearease αER positivity confers a 50 % response rate to front-line endocrine therapy, associated PR positivity, reflecting αER functionality, increases the

likelihood of favorable response to endocrine treatment by 20-30% (10). In contrast, patients who are αER and PR negative have a less than 5% response rate to endocrine treatment. Consequently, the lack of discernible ITX uptake when compared to background activity in αER+ve/PR-ve tumours may prove advantageous for patient response prediction to tamoxifen treatment. Johnston et al. previously reported that both αER+ve and αER-ve tumours progressively accumulate tamoxifen but αER+ve tumours do so much more rapidly (11). Thus, higher ITX uptake in αER+ve tumours when compared to αER-ve tumours was expected. Hypothetically, the lack of rapid ITX uptake in αER+ve/PR-ve tumours may be explained by a lower affinity of tamoxifen to non-functional αER.

Most breast tumours that acquire resistance to tamoxifen in the clinic do so in the face of a continued expression of αER. Although a variety of mechanisms for acquired tamoxifen resistance in the presence of functional αER have been put forward (12), available data in humans favor reduced intra-tumoural accumulation, possibly due to the presence of a currently unknown efflux pump. In this regard, Johnston et al. studied intra-tumoural tamoxifen accumulation matched to serum concentrations, in a total of 51 tamoxifen-resistant tumours. There was no difference in the serum concentrations of tamoxifen or N-desmethyltamoxifen in patients who developped either acquired tamoxifen resistance after a median duration of 24 months or were de novo resistant to the drug after a median of 4 months, or relapsed during adjuvant therapy after a median of 28 months. However, a significant reduction in intra-tumoural concentration of tamoxifen in patients with acquired, but not de novo, resistance was found. In some patients even a ten-fold reduction in the ratio of tumour to serum tamoxifen levels was found (13). In keeping with these findings, none of the hormone resistant metastasised patients studied showed depictable tumor ITX uptake when compared to normal breast tissue. Data from patients who have undergone serial tumour αER and PR determinations while undergoing hormonal therapy show that immunoreactive αER content does not change significantly, while PR postitivity rates drop significantly over time, in keeping with the fact that induction of PR expression requires functional αER. Thus , the findings presented again favor a lower affinity of tamoxifen to non-functional αER. Although persistant occupation of αER's by tamoxifen, taking into conisderation its long half-life in vivo, may partly explain these findings, its influence is likely to be low as all of the patients included were off antiestrogen treatment for at leats 6 months.

Due to referral limitations, no patients in whom tamoxifen treatment was going to be initiated

because of metastasis consented to participate in this study. In these patients, it would have been interesting to relate the degree of ITX uptake prior to treatment initiation to treatment outcome.

Part 2

In part 2 the rationale behind the development of agonist radioligands for visualisation of G-protein coupled gastrin-releasing peptide/bombesin receptor expressing tumours is reviewed and the human biodistribution and dosimetry in healthy volunteers of 99mTc RP527, a GRP-R agonist, as well as phase I and II studies with this ligand in patients suffering from breast carcinoma described.

It was shown that GRP receptor scintigraphy could serve several purposes, respectively : 1) to further elucidate the role of GRP/GRP-R in human maligancies, 2) to determine the incidence of functional GRP-R expression in various human maligancies in order to define diagnostic and staging imaging strategies and 3) to allow prediction of therapy responsiveness to recently developped bombesin antagonists (14).

The GRP-R agonist radioligand 99mTc-RP527 consists of a targeting peptide derived from bombesin, linked at its N-terminus via a linker group to a peptide sequence which chelates 99mTc (15). Both the unlabelled and labelled peptide bind GRP receptors with similar affinity as bombesin (IC 50 value for displacement of 125I-bombesin : 2-6 nM) and are internalized by GRP-R expressing tumours. The human biodistribution of 99mTc RP527 demonstrated low lung, myocardial and liver uptake allowing early imaging of the supradiaphragmatic region and this with a dosimetry favorable for clinical SPECT imaging (mean effective dose : 9.5^{E-03} mSv/MBq) (16). Importantly, none of the subjects studied suffered from side effects or adverse events.

Phase I/II studies showed 99mTc RP527 uptake in untreated primary breast carcinoma and related metastases, but not in metastases of previously treated patients, as well as ubiquitous and heterogeneous 99mTc RP527 uptake in non-neoplastic breast tisue. The latter finding is in keeping with available data on GRP-R expression in healthy breast tissue. Tumour activity was more easily identified on delayed images (5-6 h p.i.) rather than at 1 h p.i. due to a decrease in background activity, in keeping with the agonist character of 99mTc RP527 (17). Internalisation of the GRP/GRP-R complex involves coalescence into clathrin coated vesicles and migration to the perinuclear space. Whereas a portion of GRP-R will recycle back to the cell membrane, the ligand remains in the perinuclear space where it is trapped in lysosomes. This entrapment prolongs the residence time of GRP agonist bound radioactivity

when compared to antagonists, resulting in a higher accumulation of radioactivity in GRP-R positive tissues. The progressive and consistent increase in tumour to normal tissue ratios suggests a relative difference in clearance rate of bound and unbound radioligand from tissue and thus a specific tumour uptake mechanism in keeping with in vitro and in vivo animal data on 99mTc RP527. A GRP-R mediated uptake is further supported by the visualisation of the pancreas, a GRP-R expressing organ, in those patients in whom the pancreas was in the field of view of the tomographic images.

The different incidence numbers for GRP-R expression found by immunohistochemistry (IHC) (33%) and 99mTc RP527 imaging (88%) in the studies presented as compared to the 62% and 33% incidence respectively obtained using ^{123}I-Tyr-bombesin binding on respectively tumour homogenates and in vitro-autoradiography likely relate to differences in patient inclusion and methodology (17,18,19,20). For example, sampling errors and sub-optimal storage and assay conditions for in vitro analysis, destroying binding activity, may result in false negative IHC staining. On the other hand, with regard to imaging, peptide bombesin derivatives may also bind to neuromedin-B receptors, albeit with a much lower affinity. The latter is structurally related to the GRP-R sharing about 50 % of amino acid identity. However, there is no evidence for NMB-R expression in breast carcinoma. Additionally, the blood clearance half-life of 99mTc RP527 derived from the first exponent of the bi-exponential blood clearance curve obtained in healthy volunteers is 1.5 minutes. Consequently, binding of 99mTc RP527 to its corresponding receptors has to occur very rapidly following ligand injection which for low affinity receptors would result in a faint and probably undiscernable tumour uptake of 99mTc RP527 when compared to background activity. Future studies addressing the potential of 99mTc RP527 to visualise blockage of GRP-R following administration of a GRP-R specific targeting monoclonal antibody such as 2A11 may further confirm the presumed specific binding of 99mTc RP527 to GRP-R derived from human tumour cell line binding- and internalisation studies in vitro as well as from imaging studies in vivo in xenografted animals.

Our findings presented further suggest that in breast carcinoma GRP-R is likely expressed early in the course of disease and disappears as disease progresses, supporting a role for GRP-R as differentiation factor or morphogen. A role for GRP-R as morphogen has been previously also reported in colon carcinoma (21). In this regard, GRP is known to activate multiple different intracellular signaling pathways, including those that stimulate cell-growth, cell differentiation and cell-to cell attachment. Because in normal development many morphogens

act via heptaspanning receptors, it is not surprising that some have recently been shown to perform this role in cancer, e.g. vasoactive intestinal peptide. Larger series, however, are mandatory to confirm this finding. If correct though, bombesin antagonists e.g. MoAb 2a11 aimed at antagonising the growth stimulatory effect of the GRP/GRP-R pathway (22) are likely to be only efficacious early in the course of breast carcinoma disease.

Future prospects

The relatively low tumour to background ratios of ITX, likely related to the high volume of distribution of anti-estrogenic ligands, obviating the need for SPECT imaging may be considered a relative drawback. Hypothetically, this drawback might be overcome by pretreatment with unlabelled tamoxifen saturating the low affinity, high capacity anti-estrogenic tissue and serum binding sites thereby increasing the in-vivo availability of the radiopharmaceutical for intratumoural high affinity low capacity binding sites. In addition to testing this hypothesis, studies assessing the value of breast tumour ITX uptake for predicting response to hormonal treatment in both an adjuvant as well as palliative setting need to be carried out.

The favorable breast tumour to background ratios and incidence of 99mTc RP527 positive primary breast tumour as evidenced by the pilot study carried out warrants a comparative study with 99mTc sestamibi for scintimammography.

Provided that the preliminary findings presented, suggesting GRP-R is a differentiation factor, are confirmed, the potential usefulness of 99mTc RP527 as in vivo prognostic marker e.g. for dividing node negative breast carcinoma patients in low- or high-risk categories for recurrence, is worthy of investigation.

Finally, the predictive value of 99mTc RP527 breast tumour uptake for response to GRP-R targeted treatment options e.g. in a neo-adjuvant setting comparing chemotherapy alone versus chemotherapy plus the GRP-R treatment option could be addressed.

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SUMMARY

To maximize the benefit from routinely used e.g. triphenylethylenes and novel hormonal treatment modalities e.g. farnesyltransferase inhibitors in patients suffering from breast carcinoma , objective markers of therapeutic succes are mandatory. This could be achieved through in vivo characterization of intracellular alpha estrogen receptor (αER) and cell surface receptor status. This thesis describes both methodological and initial clinical studies with 123I-dimethyl-N,N-tamoxifen (ITX) and 99mTc RP527, radioligands for single photon emission tomographic imaging (SPECT) of human breast cancer, respectively targeted against αER and the G-protein coupled Gastrin-releasing peptide cell surface receptor (GRP-R). Results obtained with ITX are described in part I, results obtained with 99mTc RP527 are described in part II

Part I

First, it was shown that whereas available data fail to substantiate a direct relationship between breast tumour uptake of radiolabelled estradiol derivatives and αER status, in keeping with recent pathophysiological findings, sequential radiolabelled estradiol scintigraphy has the potential for early therapy response prediction to hormonal treatrment. However, as this would require two scanning episodes, an imaging technique yielding the same information in only one scanning episode is of major interest. In this regard, assessment of uptake of radiolabelled 123I-dimethyl-N,N-tamoxifen (ITX) seemed a plausible alternative.

Yang et al. previously reported high liver and lung uptake as well as progressively increasing tumour to blood ratios of ITX reaching an optimum at 24 h p.i. in breast tumour xenografts in Sprague-Dawley and Fisher 344 rats. This implied that delayed imaging may be a prerequisite to allow better depiction of ITX uptake. The human biodistribution and dosimetry study however showed low lung retention and rapid hepatobiliary excretion of ITX, resulting in excellent imaging conditions for the thoracic region even at early time points (1 h) post injection. Based on an effective dose of 8.44^E-04 (SD : 1.51^E-03 mSv/MBq), it was shown that both patients and volunteers could easily be investigated with 185 MBq ITX.

As radiolabelled metabolites not only could complicate quantitative analysis but also provide an image background that could make it difficult to identify ITX binding lesions that are small or display low ITX binding, we also studied the clearance properties and metabolite formation of ITX in human plasma. ITX was found to rapidly clear from human plasma according to a two-compartmental model with polar metabolites appearing as early as 7.5 min p.i., dominating by 20-90 min p.i. As ITX is structurally similar to tamoxifen and the CH_3I group does not interfere with the demethylation proces, the expected metabolite was N-demethyl ITX. However discordingly, the metabolite found did not display a reduction in HPLC retention time as expected. Although deaminated and hydroxylated ITX derivatives are theoretically possible, the biodistribution data in humans showing visualisation of the thyroid over time pointed towards free iodine. Given the early blood disappearance of ITX and the slow decline of metabolites, from the standpoint of imaging and quantifying ITX uptake, it could be concluded that delayed imaging is unlikely to yield better results than imaging starting at 15-30 min p.i.

Subsequently we asssessed the feasibility of ITX imaging in patients suffering from primary- and known metastatic breast carcinoma, either resistant (acquired) or non-resistant to hormonal treatment. The preferential uptake of ITX found in patients with αER and progesteron receptor (PR) positive tumours suggest that ITX imaging may prove advantageous for predicting response to tamoxifen treatment. On the other hand, the absence of ITX uptake in metastatic lesions in the setting of acguired resistance favor decreased intra-tumoural tamoxifen accumulation as a pathway for acquired resistance to tamoxifen in patients suffering from breast carcinoma. On the basis of these findings, a more extensive study examining the relationship between ITX uptake and clinical response and resistance to endocrine treatment will be initiated.

Part II

First, it was shown that GRP receptor scintigraphy could serve several purposes, respectively : 1) to further elucidate the role of GRP/GRP-R in human malignancies, 2) to determine the incidence of functional GRP-R expression in various human malignancies e.g. breast carcinoma in order to define diagnostic and staging imaging strategies and 3) to allow prediction of therapy responsiveness to recently developped bombesin antagonists.

Second, we studied the human biodistribution and dosimetry of the GRP-R agonist radioligand 99mTc RP527. 99mTc RP527 consists of a targeting peptide derived from bombesin, linked at its N-terminus via a linker group to a peptide sequence which chelates 99mTc. It was previously shown that both the unlabelled and labelled peptide bind GRP receptors with similar affinity as bombesin (IC 50 value for displacement of bombesin : 2-6 nM) and that both are internalized by GRP-R expressing tumours. The human biodistribution of 99mTc RP527 demonstrated low lung, myocardial and liver uptake

allowing early imaging of the supradiaphragmatic region and this with a dosimetry favorable for clinical SPECT imaging (mean effective dose : 9.5^{E}-03 mSv/MBq)

Third, phaseI/II studies with this ligand were performed in patients suffering from breast carcinoma. In keeping with previous in vitro autoradiography studies, 99mTc RP527 was taken up in primary-, and lymph node lesions as well as in involved bone metastases of de novo diagnosed patients. Low physiological uptake of 99mTc RP527 was also seen in normal breast tissue but this did not affect tumour depiction. In contrast, patients suffering from hormone resistant breast carcinoma failed to take up 99mTc RP527. These findings suggested that in breast carcinoma GRP-R is expressed early in the course of disease and subsequently disappears. Based on these findings, a role as morphogen for GRP-R can not be excluded.

SAMENVATTING

Ten einde onnodige en niet-efficiënte hormonale behandelingen, dit zowel voor wat gevalideerde als nieuwere medicaties betreft, te vermijden bij borstkanker patiënten zijn objectieve merkers van therapeutisch succes noodzakelijk. Potentiële kandidaat-merkers zijn radioliganden die in vivo karakterisatie van de tumorale α-estrogeen receptor (αER) en cel-oppervlak receptoren toelaten. Desbetreffende worden in deze thesis methodologische en prelimair-klinische studies beschreven met 123I-dimethyl-N,N-tamoxifen (ITX) en 99mTc RP527, SPET (single photon emission tomography) radioliganden gericht tegen respektievelijk αER en de G-proteine gekoppelde gastrin-releasing peptide receptor (GRP-R). De resultaten bekomen met ITX worden beschreven in deel I, de resulaten bekomen met 99mTc RP527 worden beschreven in deel II.

Deel I

Vooreerst wordt aangetoond dat de beschikbare literatuurgegevens een direct verband tussen tumorale opname van radioactief gemerkte estradiol-derivaten, gemeten bij middel van scintigrafie, voor beeldvorming en tumor αER status (bekomen via immunohistochemie of ligand-binding studies) niet ondersteunen. Evenwel, in overeenstemming met recente pathophysiologische bevindingen zou respons predictie op hormonale therapiëen mogelijks gerealiseerd kunnen worden door middel van sequentiële radioactief gemerkte estradiol scintigrafie. Echter dit vergt twee opeenvolgende scanning-epsioden en voor klinisch gebruik is een éénmalig onderzoek dat dezelfde informatie kan verschaffen te prefereren. In deze context leek het gebruik van radioactief gemerkt tamoxifen (ITX), een courant gebruikt anti-oestrogeen, een voor de hand liggend alternatief.

Eerder rapporteerden Yang et al. een hoge lever- en longopname evenals een progressieve toename in tumor tot bloed ITX ratios, optimaal 24 ur na radioligand injectie, na injectie van ITX in borst-tumor xenograft-bevattende Sprague-Dawley and Fisher 344 ratten. Deze bevindingen suggereerden dat laattijdige beeldvorming, teneinde ITX tumor opname beter in beeld te kunnen brengen, noodzakelijk kon zijn. Evenwel de humane biodistributie- en dosimetriestudie toonde een beperkte retentie in de longen en een snelle hepatobiliaire klaring van ITX, resulterend in excellente beeldvormingscondit012ies voor de thoracale regio vanaf 1 uur na tracer injectie. De berekende gemiddelde effectieve dosis betrof 8.44^{E}-04 (SD 1.51^{E}-03) mSv/MBq. De vooropgestelde dosis voor injectie bij patiënten aan de hand van de voorvermelde waarde is 185 MBq.

Radioactieve ITX metabolieten kunnen niet enkel de kwantitatieve analyse van ITX tumor opname bemoeilijken maar tevens een verhoging van de achtergrondactiviteit in de hand werken waardoor kleine letsels moeilijker kunnen gevisualiseerd worden. Derhalve werden de bloedklaringseigenschappen en metaboliet-vorming in humaan plasma van ITX bestudeerd. ITX werd snel geklaard uit de bloedbaan volgens een twee-compartimenteel-model waarbij polaire metabolieten verschenen in het plasma vanaf 7.5 min p.i., dominerend rond 20-90 min p.i. Aangezien ITX structureel verwant is aan tamoxifen en de CH_3I groep niet interfereert met demethylering was de meest voor de hand liggende metaboliet N-demethyl ITX. Echter, de gevormde metabolieten vertoonden niet de verwachte reductie in HPLC (high performance liquid chromatography) retentie tijd. Alhoewel gedeamineerde en gehydroxyleerde ITX derivaten theoretisch tot de mogelijkheden behoren, dient in eerste instantie gedacht te worden aan vrij iodide, mede gezien de visualisatie van de schildklier bij de gezonde vrijwilligers ondanks voorbereiding met lugol (cfr. humane biodistributie- en dosimetriestudie). Gezien de snelle bloedklaring van ITX en de trage afname van plasma ITX metabolieten kon geconcludeerd worden dat laattijdige beeldvorming géén meerwaarde zou bieden ten opzichte van vroegtijdige beeldvorming p.i.

Vervolgens werden patiënten gescand lijdend aan primair of gemetastaseerd borstcarcinoma. ITX toonde een preferentiële opname in αER en progesteron receptor (PR) positieve primaire tumor letsels dewelke een hogere respons op hormonale therapie vertonen. Verder werd er bij gemetastaseerde, tamoxifen resistente patiënten géén ITX opname in tumorletsels gevisualiseerd in overeenstemming met andere studies die efflux of sterk gereduceerde opname van tamoxifen als resistentie-mechanisme opwerpen. Deze hoopgevende bevindingen nopen tot verdere exploratie van het klinisch potentieel van ITX.

Deel II

In deel II werd eerst ingegaan op de potentiële mogelijkheden van GRP-R scintigrafie, met name : 1) verdere opheldering van de rol van de GRP/GRP-R pathway in humane tumoren, 2) het bepalen van de incidentie van GRP-R expressie in verschillende humane tumoren teneinde diagnostische en follow-up strategiëen met behulp van GRP-R radioliganden te ontwikkelen en 3) het voorspellen van respons op GRP-R antagonisten, in preklinische en klinische evaluatie.
Vervolgens werd de humane biodistributie en dosimetrie van de GRP-R agonist 99mTc RP527

beschreven. 99mTc RP527 bestaat uit een merker - eiwit afgeleid van bombesine, gekoppeld via een linker groep aan een eiwitsequentie dewelke 99mTc cheleert. Zowel het getechnetieerde als ongemerkt RP527 binden GRP-R met een vergelijkbare affiniteit als bombesine (IC_{50} waarden voor verdringing van bombesine : 2-6 nM) en worden geïnternaliseerd door GRP-R exprimerende tumoren. De humane biodistributie en dosimetrie van 99mTc RP527 toonde een lage long-, myocard- en lever opname, wat visualisatie van supradiaphragmatische tumorlokalisaties ten goede komt. De berekende gemiddelde effectieve dosis bedroeg 9.5^{E}-03 mSv/MBq

Finaal werden faze I/II studies verricht bij borstkankerpatiënten. In overeenstemming met beschikbare in vitro-autoradiografie studies werd een duidelijke 99mTc RP527 opname vastgesteld in primaire- en metastatische letsels van de novo gediagnosticeerde patiënten. 99mTc RP527 werd ook opgenomen in normaal borstweefsel, evenwel zonder dat hierdoor de detecteerbaarheid van de primaire tumor bemoeilijkt werd. Opvallend was een afwezige opname in tumor letsels van gemetastaseerde, tamoxifen resistente tumoren. Deze bevindingen sluiten een rol van GRP-R als morfogeen, waarbij in de loop van tumordedifferentiatie GRP-R verdwijnt, niet uit. Verdere exploratie evenals uitbreiding van deze bevindingen op een grotere reeks patiënten is evenwel aangewezen.

RESUME

Afin d'éviter des traitements hormonaux inutiles et inefficaces du cancer du sain, aussi bien dans l'emploi de médications validées que dans celui de nouveaux agents thérapeutiques, il nous faut rechercher des traceurs démontrant objectivement l'efficacité de l'agent thérapeutique. Des candidats traceurs potentiels sont les radio-ligands qui permettent la caractérisation in vivo des récepteurs tumoraux α-œstrogène(α-ER) et ceux de la membrane cellulaire. A cet effet nous décrivons dans cette thèse des études méthodologiques et cliniques préliminaires utilisant le 123I-dimethyl-N,N-tamoxifen (ITX) ainsi que le 99mTc-RP527, des radio-ligand SPET dirigés respectivement contre le αER et le gastrin releasing peptide récepteur (GRP-R). Les résultats obtenus avec le ITX sont décrits dans la partie I, ceux obtenus avec le 99mTc-RP527 dans la partie II.

Partie I

D'abord nous démontrons que les données de la littérature ne soutiennent pas la thèse d'un rapport direct entre la captation d'un traceur dérivé de l'estradiol (démontré par scintigraphie) et le nombre de αER (obtenus via l'immuno-histochimie ou via l'étude des liaison ligand). Cependant, conformément à de récentes constatations patho-physiologiques la prédiction de réponse à la thérapie hormonale pourrait etre réalisée au moyen de scintigraphie séquentielle de captation de l'estradiol radioactif. Cette procédure a cependant l'inconvénient de requérir deux scans consécutifs et pour l'emploi clinique un seul examen pouvant fournir les mêmes données est à préférer. Dans ce contexte l'utilisation de tamoxifen radioactif semble etre une alternative indiquée, le tamoxifen étant un anti-œstrogène d'emploi courant.

Jadis Yang et al. ont rapporté une captation élevée à hauteur du foi et des poumons ainsi qu'une augmentation progressive du rapport tumeur/sang atteignant son maximum 24h après injection du radio-ligand. L'injection du ITX fut appliquée dans un modèle de tumeur mammaire dans des rats du type Sprague-Dawley et Fisher 344. Ces constats suggéraient qu'une scintigraphie retardée pourrait etre nécessaire pour mettre en évidence la captation de l'ITX par la tumeur. Chez l'homme, par contre, l'étude de la bio-distribution et de la dosimétrie démontrèrent une rétention restreinte dans les poumons et une rapide clairance hépato-biliaire de l'ITX, avec comme effet d'excellentes images de la région thoracique dès 1 h après l'injection du traceur

La dose effective moyenne calculée s'élevait à : $8.44^{E} – 04$ (DS $1.51^{E} – 03$) mSv/MBq.

Se basant sur cette donnée la dose conseillée, injectable au patient, est de 185 MBq.

Les métabolites radioactifs de l'ITX nuisent à l'analyse quantitative de la captation tumorale et car ils procurent une augmentation du bruit de fond, ce qui rend plus difficile la visualisation de petites lésions. A cet effet nous avons étudié la clairance sanguine et la présence de métabolites de l'ITX dans le plasma sanguin. La clairance de l'ITX dans le sang est rapide et se passe selon un modèle bi-compartimental. Des métabolites polaires apparaissent dès 7.5 min après injection avec un maximum entre 20 et 90 min. L'ITX étant analogue au tamoxifen et le groupe CH_3I n'interférant point dans la déméthylation, le métabolite le plus logique est le N-démethyl ITX. Cependant, les métabolites en présence ne montraient pas la réduction escomptée du temps de rétention par HPLC (high performance liquid chromatography). Bien que les dérivés dés-aminés et hydroxylés du ITX sont théoriquement possible, il faut en premier lieu penser à l'iode libre, vu la constatation chez les volontaires sains d'une visualisation de la glande thyroïde malgré la préparation au lugol (cfr : étude de la bio-distribution et dosimétrie chez l'homme). Vu la clairance rapide du ITX et la lente décroissance de ses métabolites dans le plasma, une scintigraphie retardée ne présenterait pas d'avantage sur la scintigraphie précoce.

Ensuite des scintigraphies furent exécutées sur des malades souffrant d'un cancer mammaire primaire ou métastasé. Ici l'ITX montrait une captation préférentielle sur les récepteurs oestrogène et de la progestérone des tumeurs primaires ayant une réponse prononcée à la thérapie hormonale. De plus, chez les patients métastasés résistants au tamoxifen aucune captation de l'ITX dans les lésions ne put être visualisée, ce qui est en concordance avec d'autres études qui font état d'une captation fort réduite du tamoxifen qu'expliquerait un mécanisme de rejet.
Ces constatations prometteuses invitent à l'exploration du potentiel clinique du ITX.

Partie II

Dans la seconde partie nous examinons d'abord les possibilités potentielles de la scintigraphie du GPR-R, notamment :1) éclaircir d'avantage le rôle du système GRP/GRP-R dans les tumeurs humaines , 2) déterminer l'incidence de l'expression GRP-R dans différents types de tumeurs humaines afin de développer des stratégies diagnostiques et de follow-up au moyen de radio-ligands du GRP-R et 3) prédire la réponse aux antagonistes du GRP-R dans l'évaluation pré-clinique et clinique.

Ensuite, nous décrivons la bio-distribution et la dosimétrie du 99mTc-RP527, agoniste du

GRP-R. Le 99mTc-RP527 consiste en une protéine traceuse dérivée de la bombésine ou le 99mTc est fixé par chélation. Aussi bien la forme froide du RP527 que celle technetiée se fixent au GRP-R avec une affinité comparable à celle de la bombésine (valeur de supplantation de la bombésine IC_{50} = 2-6 nM) et sont incorporées dans les tumeurs à expression GRP-R. La bio-distribution et la dosimétrie du 99mTcRP527 chez l'homme montrent une faible captation dans les poumons, le myocarde et le foie, ce qui favorise la localisation de tumeurs supra-diaphragmale. La dose effective moyenne calculée s'élevait à 9.5^{E} – 03 mSv/MBq.

Pour finir, des examens furent fait sur des patients en phase I/II de tumeurs mammaire. En concordance avec les données d'autoradiographie in vitro une nette captation du 99mTcRP527 fut constatée dans les lésions primaires et métastasées des patients non-traités ultérieurement? Le 99mTcRP527 est également capté par le tissu mammaire sain, ceci ne gênant pas cependant la détection de la tumeur primaire. Ce qui est frappant c'est l'absence de captation dans les lésions de tumeurs métastasées résistantes au tamoxifen. Ces constatations n'excluent point un rôle de morphogène du GRP-R par lequel le GRP-R disparaîtrait au cours de la différentiation tumorale. Une plus ample exploration de ces constatations est indiquée sur une série élargie de patients.

LIST OF PUBLICATIONS

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PEER REVIEWED ARTICLES WITH INTERNATIONAL DISTRIBUTION

1992

1   Lymphocyte labelling with Tc-99m HMPAO : a radiotoxicity study using the micronucleus assay
     HMA Thierens, AM Vral, JP Van Haelst, <u>C Van de Wiele</u>, KHG Schelstraete, LF De Ridder
     J Nucl Med 1992; 33: 1167-1174

1996

2   Whole-body bone scan findings in X-linked hypophosphatemia
     <u>C Van de Wiele</u>, RA Dierckx, L Weynants, M Simons, JM Kaufman
     Clin Nucl Med 1996; 21 : 483

1997

3   Aseptic necrosis : a scintigraphic imitator of osseous involvement in Ga-67 avid
     lymphoma
     <u>C Van De Wiele</u>, RA Dierckx, L Noens, M Simons
     Clin Nucl Med 1997; 22 : 101-103

4   Delayed epiphyseal closure attributable to androgen deficiency
     <u>C Van de Wiele</u>, K Osmanagaoglu, K Audenaert, RA Dierckx
     Clin Nucl Med 1997; 22 : 567-568

5   Decreased chemotaxis of lymphocytes labelled withTc-99m HMPAO is possibly related to apoptosis
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6   Thallium-201 scintigraphy does not allow visualisation of the thyroid in euthyroid and hyperthyroid patients
     treated with amiodarone
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1998

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     breast carcinoma by SPECT
     F De Vos, <u>C Van de Wiele</u>, G Slegers and RA Dierckx
     J labelled compd rad 1998; XL: 375-376

10   Age and gender related 24 hours DMSA uptake in children with normal DMSA
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        differentiation in vivo.
        B Brans, G Laureys, V Schelfhout, C Van de Wiele, CR De Potter, C Dhooge, M Simons, RA Dierckx
        Eur J Nucl Med 1998; 25: 144-149

12   Optimizing background correction when calculating differential renal function in the presence of
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COMMUNICATIONS : INTERNATIONAL SYMPOSIA (ABSTRACTED)

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Copenhagen, 14-18 IX 1996
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Copenhagen, 14-18 IX 1996 (p)
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28   European Association of Nuclear Medicine Congress
Copenhagen, 14-18 IX 1996
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29   European Association of Nuclear Medicine Congress
Copenhagen, 14-18 IX 1996
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Copenhagen, 14-18 IX 1996
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<u>C Van De Wiele</u>, K Everaert, JP Van Haelst, H Van Der Eecken, RA Dierckx
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31   35ème Colloque de Médecine nucléaire de la langue française
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33   Benelux Symposium on Nuclear Medicine
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34   Benelux Symposium on Nuclear Medicine
Mol, 16 XI 1996
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<u>C Van de Wiele</u>, JP Van Haelst, J Philippé, J Van Damme, H Thierens, B Brans, RA Dierckx
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Mol, 16 XI 1996
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L Lambrecht, D De Neef, C Van de Wiele, G De Smet, M Troch, RA Dierckx
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36   Benelux Symposium on Nuclear Medicine
Mol, 16 XI 1996
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37   Benelux Symposium on Nuclear Medicine
Mol, 16 XI 1996
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38   8th International congress ISORBE -International society on radiolabelled blood elements
Roma, 24-27 V 1997
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39   American Society of Nuclear Medicine Congress
San Antonio, 1-5 VI 1997
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40   American Society of Nuclear Medicine Congress
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C Van de Wiele, J Philippé, JP Van Haelst, J Van Damme, H Thierens, GE Leroux-Roels, RA Dierckx
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41   American Society of Nuclear Medicine Congress
San Antonio, 1-5 VI 1997
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in visualizing ischemic infarcts
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42   XIIth International symposium on radiopharmaceutical chemistry
Uppsala, 15-19 VI 1997
Synthesis of I-123 iodoaminogluthetimide, a possible radioligand for the visualisation of the aromatase enzyme in
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F de Vos, C Van de Wiele, G Slegers, RA Dierckx
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43   European Association of Nuclear Medicine Congress
      Glasgow, 23-27 VIII 1997
      Pgp related Tc-99m MIBI uptake in human neuroblastoma cell lines
      C Van de Wiele, B , JP Van Haelst, J Philippé, C Dhooge, RA Dierckx
      Eur J Nucl Med 1997; 24: 933

44   European Association of Nuclear Medicine Congress
      Glasgow, 23-27 VIII 1997
      Influence of time interval and choice of background on Tc-99m DTPA differential renal function using double
      background correction
      C Van de Wiele, Y D'Asseler, M Koole, J De Sutter, C De Sadeleer, RA Dierckx
      Eur J Nucl Med 1997; 24: 910

45   European Association of Nuclear Medicine Congress
      Glasgow, 23-27 VIII 1997
      Evaluation of the accuracy of MRI-SPECT co-registration using 8 different algorithms
      Y D'Asseler, M Koole, Y De Deene, C Van de Wiele, R Achten, RA Dierckx, I Lemahieu
      Eur J Nucl Med 1997; 24: 887

46   European Association of Nuclear Medicine Congress
      Glasgow, 23-27 VIII 1997
      24 h absolute DMSA uptake in children with normal kidneys in relation to age and gender
      C Van de Wiele, M Simons, J Van de Walle, J De Sutter, A Raes, K Everaert, RA Dierckx
      Eur J Nucl Med 1997; 24: 867

47   European Association of Nuclear Medicine Congress
      Glasgow, 23-27 VIII 1997
      Tc-99m HMPAO labelled white blood cell scanning for the detection of infection in end-stage renal disease
      K Casier, J Versijpt, R Vanholder, C Van de Wiele, M Simons, RA Dierckx
      Eur J Nucl Med 1997; 24: 977

48   European Association of Nuclear Medicine Congress
      Glasgow, 23-27 VIII 1997
      Reproducibility of 24 h absolute DMSA uptake in children
      C Van de Wiele, S De Man, J De Sutter, J Van de Walle, M Simons, RA Dierckx
      Eur J Nucl Med 1997; 24: 1002

49   Société internationale d'urologie : 24th congress
      Montreal, 7-11 IX 1997
      Tc-99m DMSA absolute renal uptake and tubular proteinuria as parameters of severity of tubular damage following
      partial ureteral obstruction in rats
      K Everaert, C Van de Wiele, JP Van Haelst, J Delanghe, J Vande Voorde, RA Dierckx, W Oosterlinck
      Brit J Urol 1997

1998

50   International symposium on Radioactive isotopes in clinical medicine and research
      Badgenstein, 14 I 1998
      Synthesis and evaluation of I-123 aminogluthetimide, a ligand for the visualisation of the aromatase enzyme by
      SPECT
      F De Vos, G Slegers, C Van de Wiele, RA Dierckx
      Eur J Nucl Med 1998; 25: S6

51   18th annual meeting of the Belgian Society of Cardiology, Brussels, II, 1998
      Extent of scarring on perfusion imaging and incidence of appropriate shocks in patients with coronary artery disease
      and life-threatening arrythmias treated with implantable cardioverter-defibrillators.
      JH De Sutter, R Tavernier, C Van de Wiele, RA Dierckx, G De Backer, L Jordaens.
      Acta Cardiol 1998; 52: 558-559

52   18th annual meeting of the Belgian Society of Cardiology, Brussels, II, 1998
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      JH De Sutter, C Van de Wiele, Y D'Asseler, M Koole, L Bauwens, G De Backer, RA Dierckx,
      Acta Cardiol 19998; 52: 751-752

53   18th annual meeting of the Belgian Society of Cardiology, Brussels, II, 1998
      Electrocardiographic determination of infarct size after myocardial infarction treated with primary angioplasty.
      JH De Sutter, C Van de Wiele, Y Taeymans, RA Dierckx, G De Backer, DL Clement.
      Acta Cardiol 1998; 52: 573-572.

54   18th annual meeting of the Belgian Society of Cardiology, Brussels, II, 1998
      Exercise training in patients with normal and reduced ejection fraction after acute myocardial infarction treated with primary angioplasty.
      JH De Sutter, C Van de Wiele, P Gheeraert, RA Dierckx, Y Taeymans, G De Backer.
      Acta cardiol 1998; 52:573-574.

55   American College of Cardiology 47th Annual scientific session
      Atlanta, 29 III - 1 IV1998
      Value of the Selvester 32 point QRS score after acute myocardial infarction treated with primary percutaneous transluminal angioplasty
      JH De Sutter, C Van de Wiele, Y Taeymans, RA Dierckx, G De Backer, DL Clement
      J Am Coll Cardiol 1998; 31: 345A-346A

56   American College of Cardiology 47th Annual scientific session
      Atlanta, 29 III - 1 IV1998
      Effects of exercise training in patients with acute myocardial infarction treated with primary percutaneous transluminal angioplasty
      JH De Sutter, C Van de Wiele, P Gheeraert, RA Dierckx, Y Taeymans, G De Backer
      J Am Coll Cardiol 1998; 31: 365A

57   XIIIth World congress of cardiology
      Rio de Janeiro, 26-30 IV 1998
      The incidence of appropriate shocks in patients with coronary artery disease and life-threatening arythmias treated with implantable carioverter-defibrillators is determined by the extent of scarring on perfusion imaging
      JH De Sutter, R Tavernier, C Van de Wiele, RA Dierckx, G De Backer, L Jordaens
      J Am Coll Cardiol 1998; 31: 58C

58   XIIIth World congress of cardiology
      Rio de Janeiro, 26-30 IV 1998
      Quantification of myocardial infarct size with 201-thallium perfusion imaging after primary PTCA.
      JH De Sutter, C Van de Wiele, P Gheeraert, RA Dierckx, Y Taeymans, G De Backer
      J Am Coll Cardiol 1998; 31: 58C

59   International Congress of Nuclear Oncology,
      Antalya, 28 IV-4 V 98,
      Cytogenetic study of equivalent total body dose to patients treated with 131I and 131MIBG for thyroid carcinoma and neuroblastoma
      M Monsieurs, H Thierens, RA Dierckx, A Vral, C Van de Wiele, G Laureys,
      H De Winter, JM Kaufman, L De Ridder

60   45th Annual Meeting of the Society of Nuclear Medicine,
      Toronto, 7-11 VI 1998.
      201-Thallium SPECT and QT dispersion as predictors of inducibility during electrophysiological testing in patients with life-threatening ventricular arrythmias
      J De Sutter, C Van de Wiele, R Tavernier, J De Backer, G De Backer,
      L Jordaens, RA Dierckx
      J Nucl Med, 1998:; 39: 155P

61   45th Annual Meeting of the Society of Nuclear Medicine,
Toronto, 7-11 VI 1998.
Influence of growth hormone treatment on kidney function as determined by absolute 24 hours DMSA uptake in patients suffering from Turner syndrome
C Van de Wiele, S Van Aken, K Casier, C De Sadeleer, M Craen, RA Dierckx
J Nucl Med, 1998:; 39: 198P

62   45th Annual Meeting of the Society of Nuclear Medicine,
Toronto, 7-11 VI 1998.
Absolute 24h DMSA quantification in patients with severly reduced kidney function: a comparison with 51Cr-EDTA
C Van de Wiele, A Van den Eeckhaut, W Verweire, JP Van Haelst, J Versijpt, RA Dierckx.
J Nucl Med, 1998:; 39: 198P

63   45th Annual Meeting of the Society of Nuclear Medicine,
Toronto, 7-11 IV 1998.
Renal function in children with congenital solitary kidneys, a DMSA quantification study
C Van de Wiele, A Van den Eeckhaut, J Van de Walle, RA Dierckx
J Nucl Med, 1998:; 39: 199P

64   European Congress of Urology
1998
Does the urinary alpha 1 microglobulin detect uropathy? A prospective study in 500 patients
K Everaert, J Delanghe, W Buylaert, C Van de Wiele, W Kerkchaert, P Hoebeke,
RA Dierckx, B Clarysse, N Lameire, W Oosterlinck
Eur Urol 1998; 33: 81

65   European Society of Cardiology
Vienna, 23-26 VIII 1998
Admission fibrinogen concentration is a marker of final myocardial infarct size in patients with primary angioplasty
J De Sutter, C Van de Wiele, J De Backer, J Voet, M De Buyzere, P Gheeraert, RA Dierckx, Y Taeymans

66   Joint WFNM&B and EANM Congress,
Berlin, 30 VIII - 4 IX 1998
Bronchiectases in children without cystic fibrosis: value of ventilation/perfusion scintigraphy
A Van den Eeckhaut, G Villeirs, C Van de Wiele, F De Baets, P Duyck, RA Dierckx
Eur J Nucl Med 1998; 25: 1100

67   Joint WFNM&B and EANM Congress,
Berlin, 30 VIII - 4 IX 1998 (p)
Clinical usefullness of Tc-99m DTPA captopril scintigraphy in patients with a solitary kidney and suspicion of renal artery stenosis
C Van de Wiele, R Vanholder, T Verplancke, Y D'Asseler, K Casier, RA Dierckx
Eur J Nucl Med 1998; 25: 1107

68   Joint WFNM&B and EANM Congress,
Berlin, 30 VIII - 4 IX 1998
Evaluation of radioactive iodine treatment in patients with Graves' thyrotoxicosis and longstanding antithyroid drug resistancy
J Versijpt, K Casier, B Brans, C Van de Wiele, A Van den Eeckhaut, JM Kaufman, RA Dierckx
Eur J Nucl Med 1998; 25: 1121

69   Joint WFNM&B and EANM Congress,
Berlin, 30 VIII - 4 IX 1998
In-vivo adaptive response in patients treated with 131I for malignant and benign thyroid disease
M Monsieurs, H Thierens, L De Ridder, C Van de Wiele, A Vral and RA Dierckx
Eur J Nucl Med 1998; 25: 113

70   First International Congress on Spondyloarthropathies
      Gent, 22-26 IX 1998
      Bone scintigraphic (SCI) patterns in peripheral psoriatic arthritis (pPsA)
      H Zmierczak, <u>C Van de Wiele</u>, K De Vlam, RA Dierckx, H Mielants, EM Veys

71   First International Congress on Spondyloarthropathies
      Gent, 22-26 IX 1998
      The use of HIGscan in detection of spinal inflammation in ankylosing spondylitis.
      K De Vlam, <u>C Van de Wiele,</u> H Mielants, RA Dierckx, E M Veys

72   European Society of Cardiology, August 22-26, 1998, Vienna, Austria
      Admission fibrinogen concentration is a marker of final myocardial infarct size in patients treated with primary
      angioplasty.
      JH De Sutter, <u>C Van de Wiele</u>, J De Backer, J Voet, M De Buyzere, P Gheeraert, RA Dierckx, Y Taeymans.
      Eur Heart J 1998; 19: 565.

73   17th Annual session of the BWGCPE, Montgodinne, Belgium, October 3, 1998
      Value of QT parameters for the prediction of inducibility in patients with coronary artery disease and life-threatening
      ventricular arrythmias.
      JH De Sutter, Tavernier R, <u>Van de Wiele C</u>, Dierckx RA, L Jordaens.
      Acta Cardiol 1999; 54: 235.

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74   4th International Conference of Nuclear Cardiology
      Athens, 18 IV - 21 IV 1999
      Reversed redistribution (RR) after primary angioplasty: incidence and clinical follow-up.
      J De Sutter, <u>C Van de Wiele,</u> P Gheeraert, M De Pauw, G De Backer, Y Taeymans, RA Dierckx
      J Nucl Cardiol 1999; vol 6, nr. 1: S60

75   4th International Conference of Nuclear Cardiology
      Athens, 18 IV - 21 IV 1999
      Exercise training after primary angioplasty
      J De Sutter, <u>C Van de Wiele,</u> P Gheeraert, RA Dierckx, Y Taeymans, G De Backer
      J Nucl Cardiol 1999; vol 6, nr. 1: S82

76   4th International Conference of Nuclear Cardiology
      Athens, 18 IV - 21 IV 1999
      In patients with coronary artery disease (CAD) and ventricular tachycardia (VT) or feibrillation (VF), QT dispersion is
      not related to myocardial infarct (MI) size
      J De Sutter, R Tavernier, <u>C Van de Wiele,</u> J De Backer, A Van den Eeckhaut, RA Dierckx, L Jordaens
      J Nucl Cardiol 1999; vol 6, nr. 1: S25

77   4th International Conference of Nuclear Cardiology
      Athens, 18 IV - 21 IV 1999
      Infarct size determined with thallium SPECT imaging is an independent predictor of appropriate defibrillator (ICD)
      therapy.
      J De Sutter, R Tavernier, <u>C Van de Wiele,</u> G De Backer, L Jordaens, RA Dierckx
      J Nucl Cardiol 1999; vol 6, nr. 1: S25

78   4th International Conference of Nuclear Cardiology
      Athens, 18 IV - 21 IV 1999
      Residual ischemia does noet influence recurrence of ventricular arrhythmias (VA) after defibrillator (ICD)
      implantation
      J De Sutter, R Tavernier, <u>C Van de Wiele,</u> J De Backer, L Jordaens, RA Dierckx
      J Nucl Cardiol 1999; vol 6, nr. 1: S25

79   Symposium on the Frontiers in Nuclear Medicine Technology
      Mol, 6-7 V 1999
      55Co-EDTA for renal imaging using positron emission tomography (PET)
      A feasibility study
      P Goethals, A Volkaert, C Van de Wiele and RA Dierckx
      Tijdschr Nucl Geneeskd 1999; 21 (3): 102

80   9th European Congress on Biotechnology
      Brussel, 11-17 VII 1999
      Synthesis and in vitro evaluation of 123I-labelled human recombinant annexin V
      C Lahorte, F Dumont, G Slegers, C Van de Wiele, RA Dierckx

81   9th European Congress on Biotechnology
      Brussel, 11-17 VII 1999
      Challenges at the start of the third millennium: nuclear medicine imaging.
      RA Dierckx, B Brans, C Van de Wiele, G Slegers, H Thierens, I Lemahieu

82   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      Infarct size determined with 201Tl-SPECT is not related to exercise capacity in patients with coronary artery disease
      and reduced left ventricular function
      J De Sutter, F Vanheuverswyn, P De Bondt, C Van de Wiele, R Tavernier, RA Dierckx
      Eur J Nucl Med 1999; 26: 1067

83   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      Assessment of left ventricular ejection fraction (LVEF) and left ventricular volumes (LVV) by gated myocardial
      perfusion SPECT: a comparison between rest, bicycle exercise and dipirodamole stress testing in normal subjects
      P De Bondt, J De Sutter, F De Winter, L Bouwens, C Van de Wiele, RA Dierckx
      Eur J Nucl Med 1999; 26: 1076

84   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      Biodistribution and dosimetry of 123-Iodine labelled tamoxifen (ITX): an (anti)oestrogen receptor radioligand for
      therapy efficacy prediction
      C Van de Wiele, F De Vos, J De Sutter, F Dumont, G Slegers, H Thierens ,
      RA Dierckx
      Eur J Nucl Med 1999; 26: 1157

85   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      57Co-EDTA renal imaging in rats
      C Van de Wiele, P Goethals, A Volkaert, F De Winter, H Thierens, RA Dierckx
      Eur J Nucl Med 1999; 26: 1029

86   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      Apoptosis induction in lymphocytes following labelling with 99mTc-HMPAO and 99mTc-MIBI
      C Van de Wiele, J Philippé, JP Van Haelst, H Thierens, J Gunst, P De Bondt,
      RA Dierckx
      Eur J Nucl Med 1999; 26: 1191

87   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      99mTc-MIBI imaging in paediatric neuroblastoma and ganglioneuroma and its relation

to p-glycoprotein
C Van de Wiele, B De Moerloose, C Dhooge, J Philippé, F Speleman, Y Benoit, G Laureys, RA Dierckx
Eur J Nucl Med 1999; 26: 979

88   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      Evaluation of volume reduction (VR) and treatment related hypothyroidism (hT) in patients with non toxic goitre (NTG) treated with I-131
      F De Winter, H De Winter, P Van Crombrugge, C Van de Wiele, P De Bondt,
      RA Dierckx
      Eur J Nucl Med 1999; 26: 1086

89   European Association Nuclear Medicine Congress
      Barcelona, 9-13 X 1999
      Long term follow up of euthyroid goitre treated with I-131
      F De Winter, H De Winter, P Van Crombrugge, C Van de Wiele, B Brans,
      RA Dierckx
      Eur J Nucl Med 1999; 26: 1086

90   9th International Symposium of the International Society of Radiolabeled blood elements (ISORBE)
      Rio De Janeiro, 20-23 X 1999
      Synthesis, biodistribution and dosimetry studies of 123I-labelled annexin V in mice: a potential SPECT-ligand for visualisation of apoptopic cells
      C Lahorte, F Dumont, G Slegers, C Van de Wiele, RA Dierckx
      Nucl Med Comm 1999; 20: 961

91   9th International Symposium of the International Society of Radiolabeled blood elements (ISORBE)
      Rio De Janeiro, 20-23 X 1999
      Evaluation of (123I)-annexin V in normal and infarcted gods
      C Lahorte, J De Sutter, C Van de wiele, L Foubert, F De Winter, C De Cupere, Y Taeymans, G Van Nooten, G Slegers, RA Dierckx
      Nucl Med Comm 1999; 20: 948

2000

92   SASNM Millenium Congress & Autumn School 2000
      Bloemfontein, 10-14 IV 2000
      FDG-PET for the non-invasive diagnosis of orthopaedic infections
      F De Winter, C Van de Wiele, R Verdonk, D Vogelaers, K De Smet, P De Bondt, D De Clercq, RA Dierckx
      Proceeding congress 2000: 52

93   Peptide 2000 Congress
      Rome, 25-30 V 2000
      Biodistribution and dosimetry of 99mTc RP527, a gastrin releasing peptide agonist for the visualisation of GRP-receptor expressing malignances
      C Van de Wiele, F Dumont, RA Dierckx, G Slegers, SH Peers, JR Thornbock, H Thierens
      Nucl Med Commun 2000,

94   Peptide 2000 Congress
      Rome, 25-30 V 2000
      99mTc-RP 527, a gastrin releasing peptide (GRP) analogue for visualisation of GRP receptor expressing malignancies: a feasibility study
      C Van de Wiele, F Dumont, RA Dierckx, G Slegers, SH Peers, JR Thornbock, H Thierens
      Nucl Med Commun,2001

95 47th Annual Meeting of the Society of Nuclear Medicine
St-Louis, 3-7 VI 2000
FDG PET for the exclusion of infection in orthopedic prostheses
F De Winter, C Van de Wiele, R Verdonk, K De Smet, P De Bondt, RA Dierckx
J Nucl Med 2000, 41 (5): 327P

96 47th Annual Meeting of the Society of Nuclear Medicine
St-Louis, 3-7 VI 2000
FDG PET as a single technique is more accurate than the combination bone scan/white blood cell scan in chronic orthopedic infections (COI)
F De Winter, RA Dierckx, P De Bondt, D Vogelaers, R Verdonk, C Van de Wiele
J Nucl Med 2000, 41 (5): 16P

97 47th Annual Meeting of the Society of Nuclear Medicine
St-Louis, 3-7 VI 2000
Relation between defect size measurement using Cequal ® and LVEF in rest and post stress myocardial perfusion SPECT in a large population of patients with myocardial infarction
J De Sutter, P De Bondt, C Van de Wiele, F De Winter, RA Dierckx
J Nucl Med 2000, 41 (5): 91P

98 47th Annual Meeting of the Society of Nuclear Medicine
St-Louis, 3-7 VI 2000
Normal values of left ventricular ejection fraction (LVEF) and left ventricular volumes (LVV) measured by gated myocardial SPECT in women and men at rest and after bicycle or dipiridamole (DIP) stress testing
P De Bondt, J De Sutter, F De Winter, C Van de Wiele, RA Dierckx
J Nucl Med 2000, 41 (5): 159P

99 47th Annual Meeting of the Society of Nuclear Medicine
St-Louis, 3-7 VI 2000
Assessment of myocardial viability using FDG coincidence imaging: a clinical pilot study
J. De Sutter, F De Winter, C Van de Wiele, P De Bondt, Y D'Asseler, RA Dierckx
J Nucl Med 2000, 41 (5): 46P

100 47th Annual Meeting of the Society of Nuclear medicine
St-Louis, 3-7 VI 2000
FDG PET is highly accurate in the diagnosis of chronic osteomyelitis in the central skeleton
F De Winter, C Van de Wiele, D Vogelaers, R Verdonk, K De Smet, D De Clercq, RA Dierckx
J Nucl Med 2000, 41 (5): 15P

101 European Association of Nuclear Medicine Congress
Parijs, 2-6 IX 2000
FDG-PET for the non-invasive diagnosis of orthopaedic infections
F De Winter, C Van de Wiele, R Verdonk, D Vogelaers, K De Smet, P De Bondt, D De Clercq, RA Dierckx
Eur J Nucl Med 2000: 27/8

102 European Association of Nuclear Medicine Congress
Parijs, 2-6 IX 2000
HPLC determination and quantification of 123-I-N,N,-diethyl-tamoxifen and its metabolite in human plasma
F De vos, C Van de Wiele, M Vandecapelle, F Dumont, F Jacobs, G Slegers,
RA Dierckx
Eur J Nucl Med 2000: 27: 1211

103 Deutsch Reumatologen Congress
Aken, 13-16 IX 2000
Psoriasisarthritis, andere Spondyloarthropathien und rheumatoide Arthritis: ein Vergleich der szintigraphischen Präsentation
H Zmierczak, C Van de Wiele, RA Dierckx, H Mielants, EM Veys

ADDENDA

ADDENDUM 1

Brief explanation of the techniques used or referred to in the thesis.

Planar and tomographic scintigraphy (nuclear medicine imaging)

The gamma camera is a camera that allows detection of scintillations (flashes of light) produced when gamma rays interact with a sodium iodide crystal located at the front of the camera. The scintillations are detected by photomultiplier tubes, and whilst the areas of crystal seen by the tubes overlap, the location of each scintillation can be computed from the relative responses of each tube. The energy of each scintillation is also measured from the response of the tubes, and the electrical signal to the imaging computer consists of the location and the photon energy. In front of the crystal is a collimator which is made of lead with multiple elongated holes. The holes allow only gamma rays that are traveling perpendicularly to the crystal face to enter. The gamma photons absorbed by the crystal therefore form an image of the distribution of radionuclide in front of the camera. Aside from generating planar images by aquiring data with the head(s) of the camera in fixed position, the camera can also rotate around the patient to perform emission tomography. This technique relies on the acquisition of a number of equally spaced planar views (typically 32 or 64) as the camera rotates around the patient. These images are then reconstructed into tomograms using either backprojection algorithms or iterative reconstruction algorithms which estimate the original three-dimensional distribution of activity that would have resulted in the planar views actually obtained. When compared to planar imaging, tomography has a number of technical advantages which include improved image contrast because of elimination of overlying structures, and the potential for quantification of the tracer uptake.

In situ Hybridisation

In this technique, cloned DNA is labeled in vitro using nucleoside triphosphates that are conjugated to biotin (a naturally occurring prosthetic group that can be used as a molecular tag) or contain a fluorescent nucleoside derivative. The labeled DNA is placed on a microscopic slide to which chromosomes are attached; the sample is then treated to allow visualization by light microscopy of regions where the labeled DNA probes hybridizes to the chromosomes.

Polymerase Chain reaction (PCR)

PCR is a technique that uses genomic DNA that is digested into large fragments using a restriction enzyme and then is heat-denatured into single strands. Two synthetic oligonucleotides complementary to the 3'ends of the DNA segment of interest are added in great excess to the denatured DNA, and the temperature is lowered to 50-60°C. The genomic DNA remains denatured because the complementary strands are too low a concentration to encounter each other during the period of incubation, but the specific oligonucleotides, which are at a very high concentration, hybridize with their complementary sequences in the genomic DNA. The hybridized oligonucleotides then serve as primers for DNA chain synthesis, which begins upon addition of a supply of deoxynucleotides and a temperature-resistant DNA polymerase such as that from *Thermus aquaticus* (a bacterium that lives in hot springs). This enzyme, called *Taq polymerase*, can extend the primers at temperatures up to 72°C. When synthesis is complete, the whole mixture is further heated (to 95°C) to melt the newly formed DNA duplexes. When the temperature is lowered again, another round of synthesis takes place because excess primer is still present. Repeated cycles of synthesis (cooling) and melting (heating) quickly amplify the sequence of interest. At each round, the number of copies of the sequence between the primer is doubled; therefore the desired sequence increases exponentially.

Southern blotting

Southern blotting is a technique that can identify specific restriction fragments in a complex mixture of restriction fragments. First, the DNA to be analyzed is digested to completion with a restriction enzyme. The complex mixture of DNA fragments obtained as such is subjected to gel electrophoresis to separate the fragments according to size. However, many different fragments are of exactly the same length and these do not separate from each other. The restriction fragments present in the gel are subsequently denatured with alkali and transferred onto a nitrocellulose filter or nylon membrane by blotting. This procedure preserves the distribution of the fragments in the gel. The filter is then incubated under hybridization conditions with a specific radiolabeled DNA probe usually generated from a cloned restriction fragment. The DNA restriction fragment that is complementary to the probe hybridizes, and its location on the filter can be revealed by autoradiography.

Northern blotting

Northern blotting is a technique used to detect a particular RNA in a mixture of RNA's. A RNA sample, often the total cellular RNA content, is denatured by treatment with an agent (e.g., formaldehyde) that prevents hydrogen bonding between base pairs, ensuring that all the RNA molecules have an unfolded, linear conformation. The individual RNAs then are separated according to size by gel electrophoresis and transferred to a nitrocellulose filter to which the extended denatured RNAs adhere. The filter then is exposed to a labeled DNA probe and subjected to autoradiography. Because the amount of a specific RNA in a sample can be estimated from a Northern blot, the procedure is widely used to compare the amounts of a particular mRNA in cells under different conditions.

Western blotting

Western blotting or immunoblotting is a three-step procedure technique commonly used to separate proteins and then identify a specific protein of interest. The first step in an immunoblot assay is to separate a protein mixture on an SDS-polyacrylamide gel. Then, a paper-thin membrane made from nitrocellulose, which tenaciously binds most proteins, is applied to the face of the gel. When an electric field is applied, proteins are driven out of the gel and transferred to the membrane. This process is called blotting because the membrane picks up the proteins as if it were a blot impression of the gel. In the second step, the membrane is soaked in a solution of an antibody specific for the protein of interest. Only the band containing this protein binds to the antibody, forming a layer of antibody molecules. In the final step, the membrane is "developed" with an enzyme-linked antibody to identify the band containing the protein of interest.

Immunohistochemistry

Immunohistochemistry is a technique that allows visualization and identification of molecules in sectionned and mounted tissues through the usage of specific monoclonal antibodies. In general, fixed tissue sections mounted on slides are rehydrated in graded alcohols and then rinsed in a running water bath. To quench endogenous peroxidase activity, slides are preincubated in a hydrogen peroxidesolution in a light impermeable chamber. After washing and preincubation in blocking solution, a primary antibody is applied followed by tissue incubation. Subsequently, after washing, tissues are then incubated with a biotinylated anti-rabbit or ant-mouse IgG, washed and incubated again, this time with streptavidin conjugated to a peroxidase. After washing, slides are incubated with a liquid substrate chromogen system for a number of minutes allowing for identification of bound antibody.

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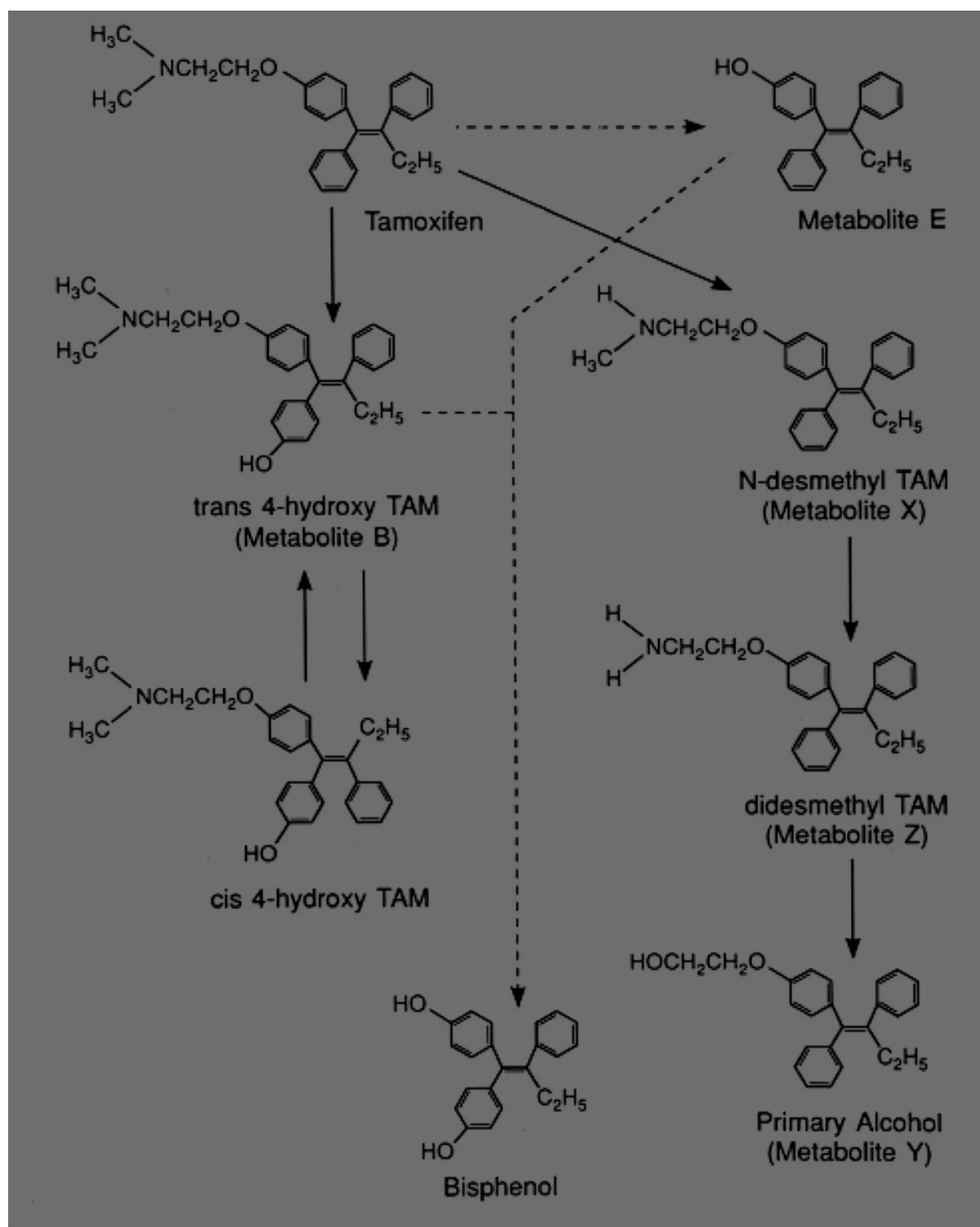
Metabolism of tamoxifen (TAM).

OCH$_2$CH$_2$N(R)$_2$

X
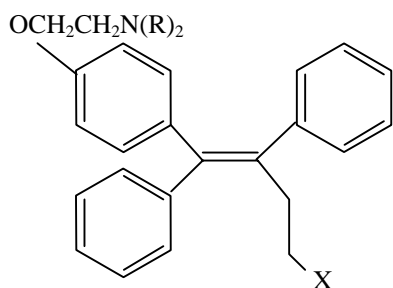
Compound	R	X
Tamoxifen	CH$_3$	H
Iodotamoxifen	C$_2$H$_5$	CH$_2$I

Structure of iodotamoxifen as compared to tamoxifen.

CURRICULUM VITAE

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Christophe Van de Wiele was born 08-10-1966 in Aalst and went to the St.-Joseph's college in the same city. He performed his studies in medicine at the University of Ghent and obtained his degree in 1991. From 1991 till 1993 he worked as a resident in internal medicine at the St.-Lucas Hospital in Brugge. From 1993 to 1994 he received training in oncology at the Jolimont Hospital, La Louvière, under the guidance of Prof. M. Bauduin followed by two years of training in nuclear medicine at the division of nuclear medicine of the University Hospital Ghent. In 1997 he was board certified in nuclear medicine. Since then he is working as a staff member at the University Hospital Ghent. His clinical responsibilities, till now, have been the development of nuclear cardiology and PET. In terms of research, both nuclear cardiology, nephrology and particularly oncology attract his main attention.

He is married to Jo Lambert and the proud father of Victor.