

Molecular markers for novel therapies in neuroendocrine (carcinoid) tumors

Judith A Gilbert^{1*}, Laura J Adhikari^{2*}, Ricardo V Lloyd², Joseph Rubin³, Paul Haluska³, Joan M Carboni⁴, Marco M Gottardis⁴ and Matthew M Ames¹

¹Department of Molecular Pharmacology and Experimental Therapeutics, ²Department of Laboratory Medicine and Pathology,

³Department of Oncology, Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905, USA

⁴Oncology Drug Discovery, Bristol-Myers Squibb, Princeton, New Jersey 08543, USA

(Correspondence should be addressed to M M Ames; Email: ames.matthew@mayo.edu)

*J A Gilbert and L J Adhikari contributed equally to this work)

Abstract

Neuroendocrine (carcinoid) tumors (NETs) are endocrine neoplasms occurring most frequently in gastrointestinal and bronchopulmonary (BP) systems. The majority of patients present with advanced disease for which few treatment options exist. We assessed 104 NETs (74 cases) for biomarkers targeted by anticancer drugs under development for other forms of cancer. Activating mutations were assessed in *epidermal growth factor receptor (EGFR)*, *stem cell factor receptor (KIT)*, and *platelet-derived growth factor receptor alpha (PDGFRA)*, as well as non-response mutations in *KRAS*. Copy number of *EGFR* and *HER-2/neu* was quantified with fluorescence *in situ* hybridization. Immunohistochemical analyses were performed for EGFR, KIT, PDGFRA, somatostatin receptor subtypes 2A and 5 (SSTR5), vascular endothelial growth factor receptor 1, mammalian target of rapamycin (mTOR), insulin-like growth factor 1 receptor (IGF1R), heat shock protein 90 (Hsp90), and transforming growth factor- β receptor 1 (TGFBR1). NETs lacked HER2-overexpression predictive of anti-HER2 response and *KIT* and *PDGFRA* activating mutations indicative of imatinib sensitivity. High *EGFR* aneusomy (20% of all cases) and elevated *EGFR* copy number (39%) were found, but few *KRAS* mutations associated with non-response to anti-EGFR therapy (3%). Hsp90, TGFBR1, IGF1R, and SSTR5 exhibited highest levels of immunohistochemical staining in the largest percents of tumors. In subsequent *in vitro* studies, anticancer drug 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) (targeting Hsp90) inhibited proliferation of BP NET lines NCI-H727, NCI-H720, and NCI-H835 with IC₅₀ values of 70.4, 310, and 788 nM respectively; BMS-754807 (targeting IGF1R/IR) inhibited growth with IC₅₀ values of 428 nM, 2.8 μ M, and 1 μ M. At growth-inhibiting concentrations, 17-AAG (24 h) induced loss of EGFR and IGF1R in the IGF1R-expressing NCI-H727 line, and BMS-754807 (24 h) inhibited constitutive IGF1R autophosphorylation. Our results support further research into Hsp90, IGF1R, and EGFR as targets for developing new anticancer therapeutics for some NETs.

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Introduction

Neuroendocrine (carcinoid) tumors (NETs) are neoplasms of the endocrine system arising predominantly in the gastrointestinal (GI) tract and respiratory system. These neoplasms originate in cells of the diffuse neuroendocrine system, and as a group display a heterogeneity related to the diversity of the sites of origin, e.g. the endocrine cells of the GI tract, the enterochromaffin-like cells of the stomach, the

Kulchitsky cells of the bronchopulmonary (BP) system. Neuroendocrine tumors characteristically overproduce biogenic amines and polypeptide hormones, with the result that advanced disease is frequently manifested clinically by the carcinoid syndrome. Surgery can be curative for small lesions, but the majority of neuroendocrine tumors are indolent and patients often present with advanced disease for which effective treatment options are limited.

NETs, long understood to express neuroendocrine biomarkers and to produce polypeptide hormones, have more recently become known for expression of several growth factor proteins (see review of Schnirer et al. (2003)). In recent years of rationally designed anticancer therapies, protein kinase inhibitors and/or antibodies directed toward growth factor receptors and downstream effectors and regulators have been under development for other forms of cancer. Targets of these new inhibitor/antibody chemotherapeutic pharmaceuticals that might play a role in the biology of neuroendocrine tumors were therefore assessed in a collection of 104 NETs that included six primary and seven metastatic tissue sites (74 cases) to evaluate their potential as biomarkers for novel therapies in these neoplasms.

Genetic abnormalities in *epidermal growth factor receptor (EGFR)*, *KRAS*, *HER-2/neu*, *platelet-derived growth factor receptor alpha (PDGFRA)*, and *stem cell factor receptor (KIT)* were assessed in neuroendocrine tumors to identify biomarkers with potential for indicating response of these neoplasms to chemotherapeutics developed against those targets in other cancers. Mutations in *EGFR* exons 18, 19, and 21 are associated in non-small-cell lung cancer (NSCLC) with response to the tyrosine kinase inhibitor (TKI) gefitinib (Lynch et al. 2004, Paez et al. 2004), whereas high *EGFR* copy number, determined by fluorescence *in situ* hybridization (FISH), is associated with sensitivity to gefitinib in NSCLC (Cappuzzo et al. 2005a, Hirsch et al. 2005) and to monoclonal antibodies cetuximab and panitumumab in colorectal cancer (Moroni et al. 2005, Sartore-Bianchi et al. 2007, Cappuzzo et al. 2008). In this study, activating mutations and FISH-determined copy number of *EGFR* (a protein expressed in 91% of GI neuroendocrine tumor primaries as well as 98% of metastases (Papouchado et al. 2005)) were investigated as markers for response to anti-EGFR inhibitors and antibodies. Mutations in *KRAS* codons 12 and 13 are associated with lack of response to cetuximab (Lievre et al. 2006, De Roock et al. 2008, Karapetis et al. 2008) and panitumumab (Amado et al. 2008) in colorectal cancer, and to TKIs gefitinib and erlotinib in lung cancer (Eberhard et al. 2005, Pao et al. 2005, Massarelli et al. 2007); therefore, *KRAS* mutations associated with non-response to anti-EGFR therapy were also assessed in NETs. High gene copy number of *EGFR* family member *HER2* predicts response to monoclonal antibody trastuzumab in breast cancer (Perez et al. 2002); thus, *HER-2/neu* copy number analysis by FISH was employed to explore *HER2*-overexpression in neuroendocrine tumors as a biomarker for sensitivity to

anti-*HER2* therapy. Finally, mutations in *KIT* exons 9, 11, 13, and 17 (Heinrich et al. 2003) and in *PDGFRA* exons 12, 14, and 18 (Corless et al. 2005) are associated with response of the gastrointestinal stromal tumor (GIST) to the TKI imatinib; as *PDGFRA* expression in neuroendocrine tumors is established (Schnirer et al. 2003), the mutational analyses of *PDGFRA* and *KIT* in these neoplasms were undertaken as predictors of imatinib effectiveness.

Immunohistochemical studies were performed to measure the expression levels of growth factor receptors and downstream effectors and regulators more recently developed as targets of anticancer drugs in other cancers, to assess their potential for indicating sensitivity in NETs. Neuroendocrine tumors exhibit vascular endothelial growth factor (VEGF) expression as well as transforming growth factor (TGF) secretion (Schnirer et al. 2003), leading us to examine VEGF receptor 1 (VEGFR1 or FLT1) immunohistochemistry (IHC) for appraising the potential of anti-angiogenesis therapy in these malignancies and TGF- β receptor 1 (TGFBR1) expression for assessing the possibilities of anti-TGFBR1 kinase inhibitors. Furthermore, in an Affymetrix (Santa Clara, CA, USA) GeneChip (Human Genome U133A) microarray analysis that compared differences in gene expression between indolent and aggressive neuroendocrine neoplasms (five pairs), ≥ 2 -fold up-regulation was found in four of five aggressive NETs for Hsp90 alpha, insulin-like growth factor binding protein 2, and the mammalian target of rapamycin (mTOR) substrate eukaryotic translation initiation factor 4E binding protein 1 (J A Gilbert, J Rubin & M M Ames 2002, unpublished observations). Therefore, expression of heat shock protein 90 (Hsp90), insulin-like growth factor 1 receptor (IGF1R), and mTOR was explored using immunohistochemical analysis in neuroendocrine tumors to allow further evaluation in these neoplasms of the role for recently developed anticancer inhibitors/antibodies targeting these proteins.

Finally, follow-up *in vitro* studies were performed in three commercially available neuroendocrine tumor cell lines (BP origin) with chemotherapeutics targeting IGF1R and Hsp90, two strongly expressed biomarkers identified immunohistochemically in all neuroendocrine tumors. The effect of anticancer drugs 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) (selective for Hsp90) and BMS-754807 (a dual inhibitor selective for IGF1R/IR) on cellular proliferation and biomarker functionality was measured.

We report here the results of a multifaceted approach (including mutational, gene copy number, immunohistochemical, and *in vitro* analyses) for

assessing recently developed targets of anticancer therapeutics for their relevance in a heterogeneous collection of neuroendocrine tumors. This work was presented in preliminary form at the 100th Annual Meeting of the American Association for Cancer Research in April 2009 (Gilbert *et al.* 2009).

Materials and methods

Patient samples

Seventy-four cases with NETs (carcinoid tumors) associated with a variety of sites were identified at Mayo Clinic Rochester, with pathology slides accessible for review as well as with both formalin-fixed, paraffin-embedded tumor blocks and flash-frozen surgical specimens available for analyses. Prior to inclusion of a case in this study, a hematoxylin and eosin (H&E) stained slide from each paraffin-embedded tissue block associated with the case was reviewed by a pathologist (R V L) to confirm the diagnosis of neuroendocrine tumor. Written research authorization was obtained from all patients for this study, in addition to Mayo Clinic Institutional Review Board approval.

Tissue microarray construction

A tissue microarray (TMA) was constructed by the Tissue and Cell Molecular Analysis Shared Resource, Mayo Clinic Rochester, with a Beecher ATA-27 automated arrayer (Sun Prairie, WI, USA). From the 74 cases, 104 NETs were selected for study from six primary and seven metastatic tissue sites. The most characteristic area from each individual NET was circled on an H&E slide, and triplicate 0.6 mm cores were removed from the corresponding area of tissue in the associated formalin-fixed, paraffin-embedded tumor block and were placed into a single recipient paraffin block. All tumor tissues included in construction of the TMA are listed, by case, in [Supplementary Table S1](#) (see section on [supplementary data](#) given at the end of this article).

Immunohistochemical analysis

Sections (5 μ) of the NET TMA were analyzed by IHC for EGFR, KIT, PDGFRA, VEGFR1, mTOR, IGF1R, Hsp90, TGFBR1, and somatostatin receptor subtypes 2A (SSTR2A) and 5 (SSTR5). Immunohistochemical staining was performed on TMA sections by the Tissue and Cell Molecular Analysis Shared Resource, Mayo Clinic Rochester. Negative controls were prepared by

substituting diluent for primary antibodies. IHC of all biomarkers was scored based on intensity from 0 to 3 by a single reviewer (L J A) and reviewed by another pathologist (R V L), with 0 indicating absence of staining, and 1, 2, and 3 representing weak, moderate, and strong staining intensity respectively. The immunohistochemical intensity score reported for the staining of tumor cells within each assessable NET sample was the average from the replicate TMA cores for that tissue. Because progenitor cells of NETs belong to the diffuse neuroendocrine system, easily accessible normal tissue of origin for use in direct comparisons of tumor immunohistochemical staining was not readily available. All IHC antibodies and epitope retrieval methods are listed in [Supplementary Table S2](#) (see section on [supplementary data](#) given at the end of this article).

FISH analysis of gene copy number

NET TMA sections (5 μ) were analyzed by FISH for *EGFR* and *HER-2/neu* copy number by the Cytogenetics Shared Resource Laboratory, Mayo Clinic Rochester, as reported (Gilbert *et al.* 2008). Thirty nuclei were scored per sample with quantitation of red signals (*HER-2/neu* or *EGFR*) and green signals (chromosome 17 centromere (CEP17) or chromosome 7 centromere (CEP7) respectively). A ratio of *HER-2/neu*:CEP17 or *EGFR*:CEP7 of 0.8–1.30 was defined as normal, <0.8 was interpreted as gene deletion, 1.30–2.0 was defined as gene duplication, and ≥ 2.0 was interpreted as gene amplification. Aneusomy was defined as a normal *HER-2/neu*:CEP17 or *EGFR*:CEP7 ratio with >30% of cells having ≥ 3 CEP17 or CEP7 signals respectively (i.e. a balanced gain in the number of gene copies and the number of chromosome copies). Aneuploid tumors were examined for the mean *EGFR* copy number/cell to determine whether *EGFR* copy number was elevated (≥ 2.47 /nucleus (Sartore-Bianchi *et al.* 2007) or ≥ 2.92 /cell (Cappuzzo *et al.* 2008)). *HER-2/neu* and *EGFR* FISH-positive samples were defined to be those demonstrating amplification or high aneusomy with $\geq 40\%$ of cells having ≥ 4 copies of *HER-2/neu* or *EGFR* respectively. Owing to the inherent admixture of tumor and non-tumor cells within the neuroendocrine tumors, the FISH-positivity for *HER-2/neu* and *EGFR* as well as the FISH-determined elevation of *EGFR* copy number/cell for each assessable neoplasm was assigned the highest value score from the replicate TMA cores for that sample.

Mutational analysis of selected *EGFR*, *KIT*, *PDGFRA*, and *KRAS* exons

One hundred and two NET surgical specimens from the 74 cases in this study were flash frozen following excision and stored at -70°C . DNA was isolated from frozen tumor samples with the QIAamp DNA Mini Kit (Qiagen), and employed for mutational analysis following amplification by the PCR. All neuroendocrine tumor tissues assessed for *EGFR*, *KIT*, *PDGFRA*, and *KRAS* mutations are listed, by case, in [Supplementary Table S1](#). Mutational analyses of *EGFR* (exons 18, 19, and 21) were performed as previously described ([Gilbert et al. 2005](#)); *KIT* mutational analyses (exons 9, 11, 13, and 17) were conducted as reported ([Gilbert et al. 2008](#)).

Mutational analyses of *KRAS* (exon 2) and *PDGFRA* (exons 12, 14, and 18) were performed following PCR with PerfeCTa qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) for *KRAS* and with iQ Supermix (Bio-Rad) with 1.25 units, final, of iTaq DNA Polymerase (Bio-Rad) for *PDGFRA*. The PCR primers and annealing temperatures for *PDGFRA* and *KRAS* are listed in [Supplementary Table S3](#) (see section on [supplementary data](#) given at the end of this article). PCR amplifications were done in a Perkin-Elmer (Foster City, CA, USA) model 9700 thermal cycler, and amplicons were sequenced on both strands by the DNA Sequencing Core, Mayo Clinic Rochester, using the universal M13 forward and reverse sequences as primers. ABI (Foster City, CA, USA) BigDye Terminator sequencing chemistry was used with an ABI 3730 DNA sequencer, and sequencing chromatograms were analyzed with Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Independent amplifications, followed by sequencing, were performed for all polymorphisms and mutations observed. GenBank accession numbers for the *PDGFRA* reference sequences used in this study were NT_022853.14 and NM_006206.3, whereas NG_007524.1 and NM_004985.3 were the reference sequences employed for *KRAS*.

Cell culture

The NCI-H727 (monolayer), NCI-H720 (suspension), and NCI-H835 (suspension) BP NET (carcinoid) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cell lines were cultured at 37°C in a humidified environment of 95%:5% air:CO₂ in RPMI 1640 (Invitrogen) supplemented with 10% v/v heat-inactivated fetal bovine serum (PAA Labs, New Bedford, MA, USA).

Cell proliferation assay

NCI-H727 (4000/well), NCI-H720 (20 000/well), or NCI-H835 (50 000/well) BP NET cells were seeded into 96-well plates (Corning, Corning, NY, USA) in 100 μl aliquots of growth medium, and incubated at 37°C for 48 h. Each drug concentration was added in 100 μl of growth medium containing 0.125% v/v DMSO to replicates of six wells; 100 μl of growth medium containing 0.125% v/v DMSO without drug was added to six wells of control cells. Following incubation at 37°C for 5 days (continuous drug exposure) for NCI-H727 cells (6 days for NCI-H720 and NCI-H835 cells), cell growth was determined with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay ([Cory et al. 1991](#)), by addition to each well of 40 μl containing a 20:1 ratio of 2 mg/ml MTS (Promega) and 0.92 mg/ml phenazine methosulfate (Sigma). After incubation at 37°C for 2 h, absorbance was measured at 490 nm on an MR5000 (Dynatech, Chantilly, VA, USA) or SpectraMax Plus³⁸⁴ (Molecular Devices, Sunnyvale, CA, USA) microplate reader. Three dose-response experiments for each drug were performed with each cell line, with growth of treated cells at each drug concentration compared to proliferation of control cells. IC₅₀ values were estimated with the Prism program (GraphPad, San Diego, CA, USA) by fitting the mean percent control values for each drug concentration to the best sigmoidal dose-response curve using non-linear least squares regression analysis. Anticancer drugs BMS-754807 and 17-AAG were kindly provided by Dr Scott Kaufmann, Mayo Clinic Rochester, and the National Cancer Institute (Bethesda, MD, USA) respectively.

Western immunoblot analysis

Whole cell lysates were prepared from cultured BP NET cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol; protein was quantitated with the DC Protein Assay (Bio-Rad) following the manufacturer's instructions. Samples for western blot analysis were loaded on the basis of protein concentration and subjected to electrophoresis on 10% w/v separating gels (Criterion XT, Bio-Rad). Following transfer to PVDF membranes (Bio-Rad), immunoreactive proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and HyBlot CL film (Denville Scientific, Metuchen, NJ, USA). Primary antibodies employed were anti-EGFR (Santa Cruz sc-03), anti-IGF1R β (Cell Signaling 3027; Danvers, MA, USA),

anti-phospho-IGF1R β (Tyr1131)/insulin receptor β (Tyr1146) (Cell Signaling 3021), anti-Hsp90 (Cell Signaling 4874), and anti- β -actin (Santa Cruz sc-130656); the secondary antibody was goat anti-rabbit IgG-HRP (Santa Cruz sc-2004).

Results

Patient cases

Forty-two of the 74 patients in this study were females; 32 were males (Table 1). The median age at the time of this surgery was 60 years (range 24–80). The majority of patients (57) had metastatic or multicentric disease at surgery; of this subset, 19 patients had received a variety of treatments for carcinoid disease prior to this surgery (Table 2 lists these cases and the types of therapy). Patients presenting with solitary, non-metastatic primaries at the time of surgery had BP (15) or appendiceal (2) neoplasms.

One hundred and four neuroendocrine tumors from these 74 cases were chosen for inclusion in the TMA construction, with equal numbers of primary and metastatic neoplasms (see Table 1). The BP system and small bowel were the most common primary tumor sites represented, with the liver being the most frequent metastatic tissue. Supplementary Table S1 lists all neuroendocrine tumor tissues, by case, included in the TMA.

Table 1 Patient characteristics (74 cases)

Sex	
Male	32
Female	42
Median age (range) at surgery	60 years (24–80)
Year of surgery	
1990–1999	49
2000–2009	25
History of other cancer	8
Metastatic/multicentric	57
Previous therapy	19
Primary sites in TMA ^a	
Lung	22
Small bowel	18
Colon	5
Appendix	3
Stomach	3
Pancreas	1
Metastatic sites in TMA ^a	
Liver	30
Lymph node	10
Mesentery	8
Uterus	1
Ovary	1
Peritoneum	1
Rectum	1

^aTotal of 104 NET samples in TMA.

Mutational analysis of *EGFR*, *KIT*, *PDGFRA*, and *KRAS*

A total of 102 neuroendocrine tumors from the 74 cases in this study were analyzed for mutations in selected exons of *EGFR*, *KIT*, *PDGFRA*, and *KRAS*. Supplementary Table S1 lists all tumors, by case, subjected to mutational analysis; in 36% of cases, differing numbers of neuroendocrine tumor sites were available for mutational analysis as for TMA inclusion. However, for all but three of the 74 cases, a minimum of one tumor site per case was subjected to all assays: FISH and IHC assessments (via TMA inclusion) as well as mutational analyses.

EGFR, *KIT*, and *PDGFRA*

No *EGFR* mutations predictive of gefitinib sensitivity were found in any of the 102 neuroendocrine tumor sites analyzed (81 of these analyses had been previously reported (Gilbert *et al.* 2005)). Similarly, no *KIT* or *PDGFRA* mutations associated with clinical response to imatinib were detected in any neuroendocrine tumor.

Results of mutational analyses indicated that all neuroendocrine tumors assessed from all 74 patients carried the synonymous single nucleotide polymorphism (SNP) dbSNP rs1873778 in *PDGFRA* exon 12 (A1701G, where number ‘1’ is assigned to the ‘A’ in the translation initiation codon of the cDNA; amino acid position 567), with two cases being heterozygous and 72 cases being homozygous for this SNP. Tumors from 20% of the cases harbored the synonymous SNP dbSNP rs2228230 in *PDGFRA* exon 18 (C2472T; amino acid 824), with this polymorphism being heterozygous in 13 cases and homozygous in two cases.

KRAS

KRAS codon 12 mutations associated with non-response to anti-EGFR monoclonal antibodies (Lievre *et al.* 2006, Amado *et al.* 2008, De Roock *et al.* 2008, Karapetis *et al.* 2008) and TKIs (Eberhard *et al.* 2005, Pao *et al.* 2005, Massarelli *et al.* 2007) were identified in only two neuroendocrine tumors, both aggressive neoplasms: G35A (encoding G12D) in a colon primary from a patient with a survival period of 3 months following diagnosis, and G35T (encoding G12V) in an ovarian metastasis from a patient with a 2.3-year survival (Table 2 indicates the two tumors harboring *KRAS* non-response mutations).

Mutational analysis of *KRAS* exon 2 detected the non-classical codon 18 mutation C53A (Morgan *et al.* 2001) (encoding amino acid substitution A18D) in one

Table 2 Neuroendocrine tumors exhibiting strong biomarker expression

Case	Primary site	Tissue analyzed	Hsp90	TGFBR1	IGF1R	SSTR5	PDGFRA	VEGFR1	EGFR	SSTR2A	mTOR	KIT	Mean EGFR ≥ 2.47 /cell	EGFR FISH
1 ^{a,b}	Small bowel	Lymph node	- ^c	+ ^c	+	-	-	-	-	-	-	-	-	-
2	Lung	Lung	-	+	+	-	+	-	-	-	-	-	-	-
3	Lung	Lymph node	+	+	-	-	-	-	-	-	-	-	-	-
4	Lung	Lung	+	+	+	+	+	+	+	+	+	+	+	+
5	Lung	Lung	+	+	+	+	+	+	+	+	+	+	+	+
6	Small bowel	Liver	+	+	+	+	-	-	-	-	-	-	-	-
7	Lung	Lung	+	+	-	+	+	+	+	+	+	+	+	+
8	Lung	Lung	+	+	+	+	+	+	+	+	+	+	+	+
9	Small bowel+ colon	Small bowel	+	-	-	-	-	-	-	-	-	-	-	-
10	Small bowel	Liver	+	+	+	+	+	-	-	+	-	-	-	-
11	Small bowel	Small bowel	+	+	+	+	+	-	-	+	-	-	-	-
12 ^a	Lung	Liver	+	+	+	+	+	+	+	-	-	-	+	+
13 ^a	Small bowel	Mesentery	+	+	+	+	+	-	-	-	-	-	+	+
14	Small bowel	Small bowel	-	-	+	-	-	-	-	+	-	-	-	-
15	Small bowel	Liver	+	-	+	-	-	-	-	+	-	-	-	-
16 ^b	Small bowel	Small bowel	-	-	+	+	-	-	-	+	-	-	+	-
17 ^d	Lung	Lymph node	-	-	+	-	-	-	-	-	-	-	-	+
18 ^{b,d,e}	Lung	Liver	-	+	-	+	+	+	+	+	-	-	+	-
19	Lung	Liver	+	+	-	-	-	-	-	-	-	-	+	-
20	Lung	Lung	+	+	+	+	+	+	-	-	-	-	-	-
21	Lung	Lung	+	-	+	-	+	+	-	-	-	-	-	-
22 ^b	Small bowel	Liver	+	+	+	-	-	+	+	-	-	-	+	+
23 ^{b,d}	Small bowel	Small bowel	+	-	-	+	-	-	-	-	-	-	+	-
24 ^d	Small bowel	Lymph node	-	-	+	-	-	-	-	-	-	-	-	-
25 ^b	Unknown	Small bowel	-	-	+	-	-	+	-	-	-	-	-	-
26	Small bowel	Liver	-	-	+	-	-	-	-	-	-	-	-	-
27	Small bowel	Small bowel	-	-	+	-	-	+	-	-	-	-	+	+
		Mesentery	+	-	+	+	-	-	-	-	-	-	+	+
		Liver	-	-	+	-	-	-	-	-	-	-	+	+
28 ^f	Colon	Liver	-	+	-	-	-	-	-	-	-	-	+	+

Table 2 continued

Case	Primary site	Tissue analyzed	Hsp90	TGFBR1	IGF1R	SSTR5	PDGFRA	VEGFR1	EGFR	SSTR2A	mTOR	KIT	Mean EGFR ≥ 2.47 /cell	EGFR FISH
29	Small bowel	Lymph node	+	+	+	-	-	-	-	-	-	-	-	-
30	Small bowel	Peritoneum	+	+	-	+	-	-	-	-	-	-	-	-
31 ^{a,b,d,g}	Small bowel	Mesentery	+	+	+	+	-	-	-	-	-	-	-	-
32	Lung	Ovary	-	+	-	+	+	+	-	-	-	-	+	-
33	Lung	Lymph node	+	+	+	-	-	-	-	-	+	-	+	-
34	Small bowel	Lung	+	+	+	+	-	-	-	-	-	-	-	-
35	Lung	Small bowel	-	-	+	-	-	-	-	-	-	-	+	-
36 ^b	Lung	Liver	+	-	-	-	-	-	-	-	-	-	-	-
37 ^f	Small bowel	Liver	+	+	-	+	-	-	-	+	-	-	+	+
38	Small bowel	Small bowel	-	+	-	+	-	-	-	-	-	-	+	-
39	Small bowel	Liver	+	-	+	-	+	-	-	-	-	-	+	+
40	Small bowel	Liver	+	+	+	+	-	-	-	+	-	-	+	-
41 ^b	Small bowel	Liver	-	-	-	-	-	-	-	-	-	-	-	+
42 ^f	Small bowel	Liver	-	+	-	-	-	-	-	-	-	-	+	+
43	Small bowel	Small bowel	+	-	-	+	-	-	-	-	-	-	-	-
		Mesentery	+	+	+	+	-	-	-	+	-	-	-	-
44	Small bowel	Liver	+	+	-	+	+	+	-	+	-	+	-	-
		Small bowel	+	+	+	+	-	-	-	-	-	-	-	-
45	Lung	Liver	+	+	+	+	+	+	-	-	+	-	-	-
46	Lung	Lung	+	+	-	-	-	-	+	-	-	-	-	-
47	Lung	Lung	-	+	-	-	-	-	+	-	-	-	+	-
48	Lung	Lung	+	+	+	+	-	+	-	-	-	-	-	-
49	Lung	Lung	+	+	+	+	+	+	-	+	-	-	+	+
50 ^f	Lung	Lung	-	+	-	+	-	-	-	-	-	-	+	+
51	Lung	Lymph node	-	-	-	-	-	-	-	-	-	-	+	+
52	Lung	Lung	-	+	+	-	-	-	-	-	-	-	-	-
53 ^g	Colon	Colon	+	+	-	+	+	+	-	-	+	-	+	+
54	Lung	Lung	+	-	-	-	-	-	-	-	-	-	+	+
55	Lung	Lung	+	+	+	-	-	+	-	-	-	-	+	+
56 ^b	Small bowel	Mesentery	-	+	-	+	+	+	-	-	-	-	+	-
57 ^b	Small bowel	Small bowel	-	+	+	+	+	+	-	-	-	-	+	-
		Lymph node	+	+	-	+	+	+	-	-	-	-	+	+

continued

Table 2 continued

Case	Primary site	Tissue analyzed	Hsp90	TGFBR1	IGF1R	SSTR5	PDGFRA	VEGFR1	EGFR	SSTR2A	mTOR	KIT	Mean EGFR ≥2.47/cell	EGFR FISH
58	Small bowel	Liver	+	+	-	+	-	-	-	-	-	-	+	+
	Small bowel	Small bowel	+	+	-	+	-	-	-	+	-	-	-	-
	Liver	Liver	+	+	+	-	-	+	-	+	-	-	-	-
59 ^d	Small bowel	Mesentery	-	-	-	-	-	-	-	-	-	-	-	-
	Small bowel	Liver	+	+	+	-	-	-	-	-	-	-	-	-
60	Small bowel	Mesentery	+	+	-	-	-	-	-	-	-	-	-	-
	Small bowel	Liver	+	+	-	-	-	-	-	-	-	-	-	-
61 ^b	Small bowel	Small bowel	-	-	-	-	-	-	-	-	-	-	-	-
	Rectum	Rectum	-	+	-	+	-	-	-	-	-	-	-	-
	Liver	Liver	-	+	-	-	-	-	-	-	-	-	-	-
62	Small bowel	Small bowel	+	-	-	-	-	-	-	-	-	-	-	-
63	Stomach	Stomach	-	+	-	-	+	-	-	-	-	-	+	-
	Liver	Liver	+	+	-	-	-	-	-	-	-	-	+	-
64	Stomach	Stomach	+	+	-	+	-	-	-	-	-	-	+	+
	Liver	Liver	+	+	-	+	+	-	-	+	-	-	+	+
65	Small bowel + colon	Colon	+	+	+	+	-	-	-	-	-	-	+	+
66	Colon	Colon	-	+	+	+	-	+	-	-	-	-	-	-
	Lymph node	Lymph node	+	+	-	-	-	-	-	-	-	-	-	-
67 ^d	Appendix	Appendix	+	+	+	-	+	-	-	-	-	-	-	-
	Uterus	Uterus	+	+	+	-	+	+	+	-	-	-	-	-
68	Colon	Colon	-	-	+	-	-	-	-	-	-	-	-	-
69 ^d	Small bowel	Mesentery	-	-	-	-	-	-	-	-	-	-	-	-
70	Colon	Colon	+	-	-	-	-	-	-	-	-	-	-	-
	Lymph node	Lymph node	-	-	-	-	-	-	-	-	-	-	-	-
71	Small bowel	Small bowel	+	-	-	+	-	-	-	-	-	-	-	-
72	Duodenum + stomach + pancreas	Stomach	+	+	-	+	-	+	-	-	-	-	+	-
73	Appendix	Pancreas	+	+	-	+	-	+	-	-	-	-	+	+
74	Appendix	Appendix	-	-	-	-	-	-	-	-	-	-	-	-
	Appendix	Appendix	-	-	+	-	-	-	-	-	-	-	-	-

^aNET therapy prior to this instance of surgery: chemotherapy.

^bNET therapy prior to this instance of surgery: somatostatin analog therapy (duration, 1 week-6 years).

^cFor Hsp90, TGFBR1, IGF1R, SSTR5, PDGFRA, VEGFR1, EGFR, SSTR2A, mTOR, and KIT, '+' indicates an IHC intensity score of 3, and '-' indicates an IHC score <3.

^dNET therapy prior to this instance of surgery: surgery.

^eNET therapy prior to this instance of surgery: radiation therapy.

^fTumor(s) was HER-2/neu FISH-positive.

^gTumor harbored KRAS codon 12 mutation.

neuroendocrine tumor, a liver metastasis from an ileal primary lacking this mutation. All three *KRAS* mutations found in the neuroendocrine tumors were heterozygous.

FISH analysis of *EGFR* and *HER-2/neu* copy number

EGFR

FISH analyses detected that 39% of all assessable cases had neuroendocrine tumors displaying *EGFR* aneusomy with *EGFR* copy number at the elevated levels associated with sensitivity of colorectal cancer to panitumumab (mean copy number ≥ 2.47 /nucleus (Sartore-Bianchi *et al.* 2007)); 26% exhibited aneusomy with elevated *EGFR* copy number predictive of cetuximab response (mean copy number ≥ 2.92 /cell (Cappuzzo *et al.* 2008)). Of the primary neuroendocrine tumors examined by FISH, those exhibiting elevated mean *EGFR* copy number (≥ 2.47 /nucleus) included 38% of the BP primaries and 29% of the small bowel primaries. Of 20 cases in which multiple tumor tissues from the same patient were assessable by FISH for elevated *EGFR* copy number, the primary and metastatic tumor(s) were both positive in five cases and both negative in ten patients, multiple primaries from different tissue sites were both positive in one case, multiple metastases from different sites were both negative in one case, the metastatic tumors but not the primary were positive in one case, and the primary but not the metastatic tumor(s) was positive in two cases. Table 2 indicates the neuroendocrine tumors with elevated *EGFR* copy number, on a case-by-case basis.

Fifty-two percent of cases with tumors displaying elevated *EGFR* copy number were also ‘FISH-positive’, exhibiting copy number at the high levels associated with gefitinib response (aneusomy with $\geq 40\%$ of cells having ≥ 4 copies of *EGFR* (Cappuzzo *et al.* 2005a, Hirsch *et al.* 2005)). Of these 14 patients (20% of all assessable cases) exhibiting *EGFR* FISH-positive NETs, six were identified with FISH-positive primaries (two colon, two lung, one stomach, and one pancreas primary case – with one lung and one stomach primary tumor associated with a FISH-positive metastasis). Six cases had only (FISH-positive) liver metastases available for analysis (four originating in small bowel and one each from lung and colon). The remaining two cases had FISH-negative small bowel primaries with FISH-positive liver metastases (see Table 2 for a listing of *EGFR* FISH-positive tumors).

HER-2/neu

HER-2/neu aneusomy was detected in 21% of all assessable cases, but *HER-2/neu* copy number was below levels predictive of anti-HER2 response (defined as > 6 *HER-2/neu* copies/cell or a *HER-2/neu*:CEP17 ratio > 2.2 , by FISH analysis (Wolff *et al.* 2007)). However, four cases with *HER-2/neu* aneusomy exhibited high copy number for both *EGFR* and *HER-2/neu* (aneusomy with $\geq 40\%$ of cells having ≥ 4 gene copies), a pairing predictive of gefitinib sensitivity greater than that for patients with high *EGFR* copy number alone (Cappuzzo *et al.* 2005b). Three of these four cases had only liver metastases available for analysis, one originating from an aggressive colon neoplasm and two from small bowel primaries (one multicentric); the fourth *EGFR* and *HER-2/neu* FISH-positive tumor was an atypical BP NET (associated with a FISH-positive lymph node). Table 2 indicates the *HER-2/neu* FISH-positive tumors.

Immunohistochemical analysis

Immunohistochemical staining in neuroendocrine tumors for *EGFR*, *KIT*, *PDGFRA*, *SSTR2A*, *SSTR5*, *VEGFR1*, *mTOR*, *IGF1R*, *Hsp90*, and *TGFBR1* is illustrated in Fig. 1. The immunohistochemical staining intensity score for each assessable NET in the TMA is presented for each biomarker in Fig. 2, with scores grouped by location of malignancy into one of six large categories (three for primaries and three for metastases). The biomarkers for which the largest number of neuroendocrine tumors exhibited the strongest IHC staining were *Hsp90*, *TGFBR1*, *IGF1R*, and *SSTR5*, with intensity scores of 3 for 61, 60, 53, and 51% of all neoplasms respectively. For the two most common primary sites studied, the percent of tumors which exhibited the highest *Hsp90*, *TGFBR1*, *IGF1R*, and *SSTR5* score was 74, 79, 62, and 55% of BP neoplasms respectively, and 50, 28, 44, and 61% of small bowel NETs. The biomarker with the lowest expression level by IHC was *KIT*, with complete absence of staining in 61% of all neuroendocrine tumors. For each immunohistochemical target protein studied, the staining intensity score averaged over all primaries was not significantly different from the score averaged over all metastases (data not shown) except for *mTOR*, whose average score was 1.85 ± 0.08 (mean \pm s.e.m.) for primaries and 1.44 ± 0.10 for metastases ($P < 0.01$).

Table 2 presents, by case, all assessable neuroendocrine tumors which exhibited strong immunohistochemical staining (intensity score of 3) for the ten molecular markers studied. *Hsp90*, *TGFBR1*, *IGF1R*,

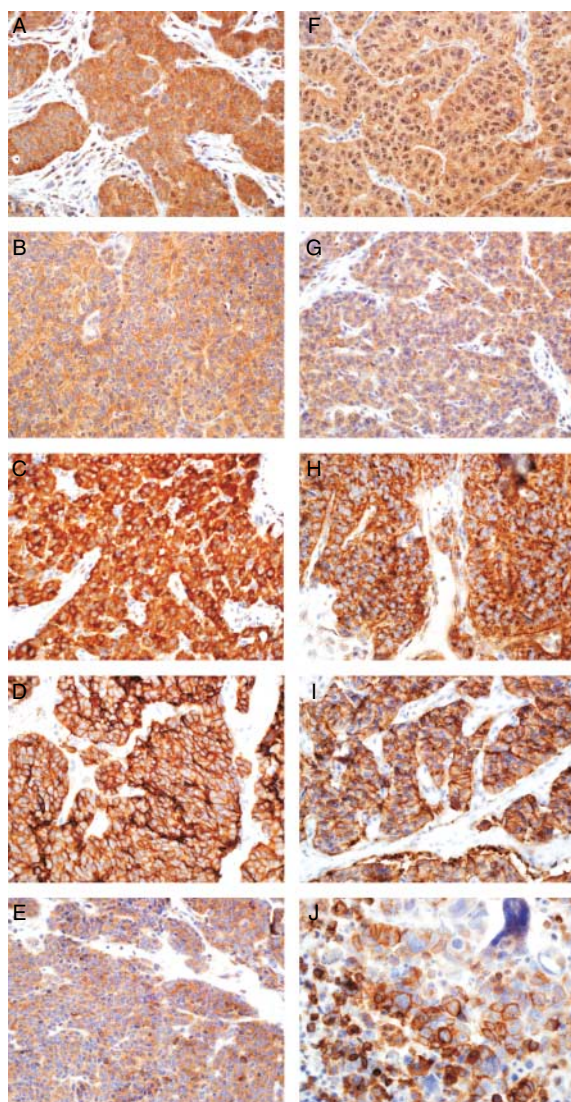


Figure 1 Immunohistochemical analysis of neuroendocrine tumors. Panels illustrate individual NET specimens in the TMA (original magnification $\times 200$) exhibiting strong immunostaining for (A) Hsp90, (B) IGF1R, (C) PDGFRA, (D) EGFR, (E) mTOR, (F) TGFBR1, (G) SSTR5, (H) VEGFR1, (I) SSTR2A, and (J) KIT.

and SSTR5 (the strongest staining biomarkers for the largest number of NETs) were expressed in all tumors from 20 cases in which primary and metastatic tumor tissues from the same patient were assessable. For the majority of these cases, tumors at all sites were consistent in expression of the four biomarkers, with all neoplasms exhibiting an IHC score of either 3 or <3 . However, a difference in immunohistochemical staining levels between primary and metastatic tumor(s) from the same patient was seen for Hsp90, TGFBR1, IGF1R, and SSTR5 in 30, 25, 45, and 40% of these cases respectively.

In vitro effects of anticancer drugs in neuroendocrine tumor cell lines

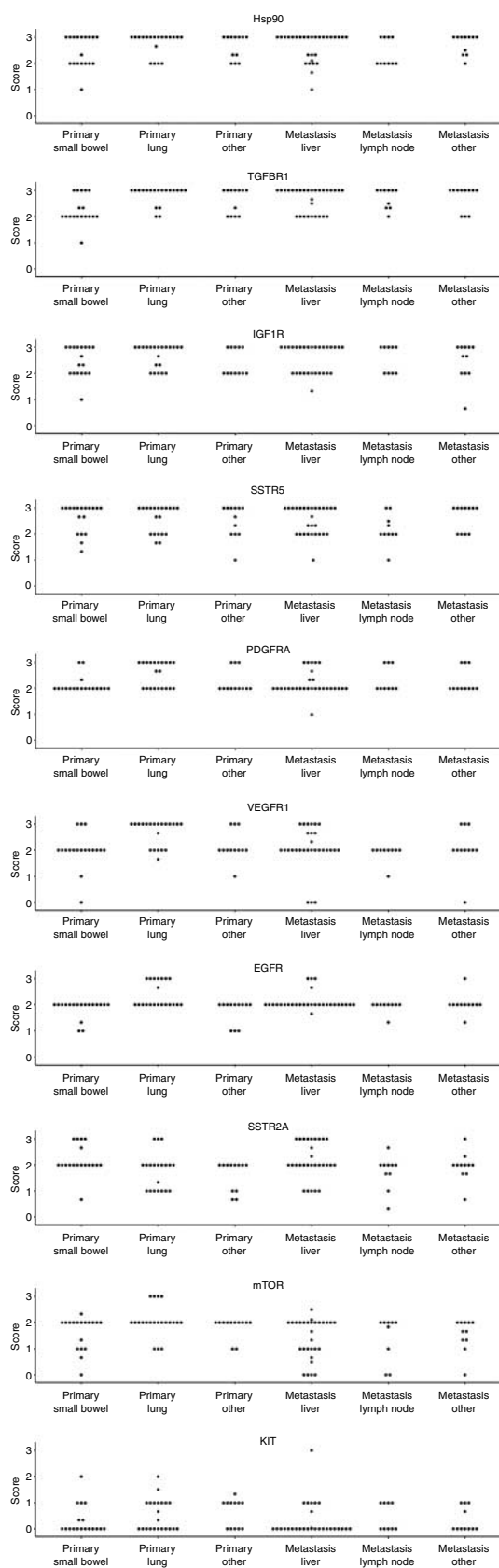
Commercially available human neuroendocrine tumor lines were obtained for *in vitro* studies assessing the effect on NET cells of anticancer drugs targeting Hsp90 and IGF1R, two of the biomarkers found by IHC to be strongly expressed in all neuroendocrine tumors. Purchasable NET lines were all of BP origin, so NCI-H727, NCI-H720, and NCI-H835 cells were employed for our studies measuring the effect of chemotherapeutics on cell proliferation and biomarker functionality.

Western immunoblot analyses provided evidence that Hsp90 was ubiquitously expressed in all three lines (Fig. 3A). MTS assays measuring the effect of the chemotherapeutic agent 17-AAG (selective for Hsp90) on BP NET cell lines indicated that 17-AAG inhibited proliferation of NCI-H727, NCI-H720, and NCI-H835 cells with IC_{50} values of 70.4, 310, and 788 nM respectively ($n=3$).

Of the three cell lines, only NCI-H727 cells expressed IGF1R at levels detectable by western immunoblot analysis (Fig. 3A). MTS assays assessing the effect of anticancer therapeutic BMS-754807 (a dual inhibitor selective for IGF1R/IR) indicated that BMS-754807 inhibited proliferation of NCI-H727, NCI-H720, and NCI-H835 cells with IC_{50} values of 428 nM, 2.8 μ M, and 1 μ M respectively ($n=3$).

EGFR aneusomy was detected by FISH analyses in all three BP NET cell lines, with elevated *EGFR* copy number predictive of sensitivity to panitumumab and cetuximab; all three lines were also *EGFR* FISH-positive, exhibiting the high levels of *EGFR* aneusomy associated with gefitinib response. However, only NCI-H727 cells expressed *EGFR* detectable by immunoblot analyses (Fig. 3A). Therefore, subsequent experiments with NET cells measuring by western analysis the downstream effects of anticancer drugs on *EGFR* and IGF1R expression, as well as on IGF1R function, were performed exclusively with the NCI-H727 line.

Incubation of NCI-H727 cells (24 h) with 17-AAG concentrations ranging from 31 to 1000 nM resulted in a dose-dependent decrease in the level of both IGF1R and *EGFR* as measured by western immunoblot analysis, with the more dramatic effect being observed on IGF1R ($n=3$). Figure 3B illustrates in NCI-H727 cells the 17-AAG-induced dose-dependent decrease in cell proliferation and in cellular levels of *EGFR* and IGF1R. Exposure (24 h) of NCI-H727 cells to BMS-754807 at concentrations from 0.3 to 10 μ M completely inhibited the constitutive autophosphorylation of IGF1R at all concentrations tested ($n=3$).



An illustration of the inhibition induced by increasing BMS-754807 concentrations on NCI-H727 cell proliferation as well as on constitutive IGF1R autophosphorylation is presented in Fig. 3C.

Discussion

While somatostatin therapy is effective for clinical symptoms associated with metastatic disease and may delay progression of disease, neuroendocrine tumors are often unresponsive to conventional chemotherapeutic regimens (see review of Schnirer *et al.* (2003)), and new drug therapies are needed. We analyzed a heterogeneous collection of NETs for genetic abnormalities as well as protein expression levels of a variety of growth factor receptors and downstream effectors and regulators targeted by anticancer drugs currently under development for other forms of cancer but whose natural ligands might also play a role in the biology of neuroendocrine tumors (see review of Schnirer *et al.* (2003)).

Mutational analyses detected neither *EGFR* mutations predictive of gefitinib sensitivity in any of the neuroendocrine tumors analyzed nor *KIT* or *PDGFRA* activating mutations predictive of imatinib response. Few *KRAS* mutations associated with non-response to anti-EGFR therapy were detected in NETs, occurring in only 3% of all cases.

FISH assays detected no *HER2* overexpression predictive of trastuzumab response in any of these malignancies; however, four cases with high *HER-2/neu* copy number also exhibited high *EGFR* copy number, a pairing predictive of gefitinib sensitivity. Elevated *EGFR* copy number was detected by FISH in 39% of all assessable cases with high aneusomy exhibited by 20%, although moderate *EGFR* expression levels were displayed immunohistochemically across all neuroendocrine tumors. Elevated *EGFR* copy number was demonstrated in both BP and GI primaries (38 vs 29%), suggesting further research into the role of anti-EGFR TKIs and monoclonal antibodies in neuroendocrine tumors.

Protein expression levels of ten biomarkers were compared immunohistochemically, including those for *SSTR2A* and *SSTR5*. The moderate expression of *SSTR2A* (the long form of *SSTR2*) in this entire

Figure 2 Immunohistochemical staining intensity of neuroendocrine tumors for ten biomarkers. Individual IHC staining intensity scores for all NETs assessable in the TMA ($n=104$) were grouped by tissue site into one of six large categories for comparison. Primary Other: colon, appendix, stomach, and pancreas primaries; Metastases Other: mesentery, uterus, ovary, peritoneum, and rectum metastases.

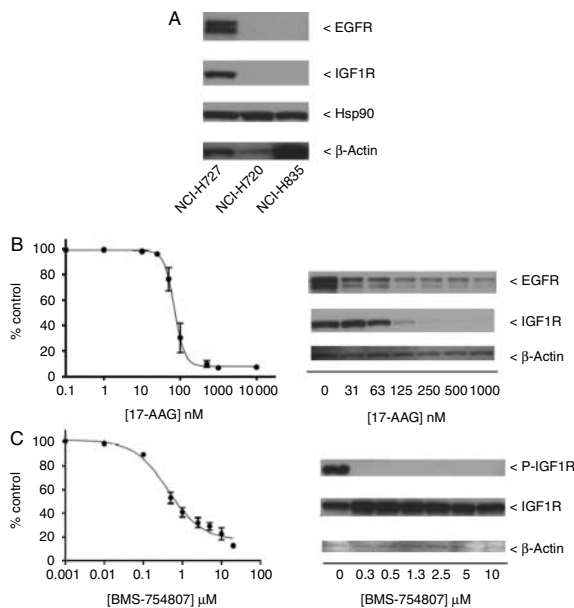


Figure 3 Effects of anticancer drugs in BP NET cell lines. (A) Whole cell lysates prepared from three BP NET cell lines (NCI-H727, NCI-H720, and NCI-H835) were analyzed by western immunoblotting for the presence of selected biomarkers. (B) Left: NCI-H727 cells (4000/well) were incubated (continuous exposure, 5 days) in 96-well plates at 37 °C with increasing concentrations of the anticancer drug 17-AAG (targeting Hsp90) in serum-containing medium, with cell proliferation determined by the MTS assay; right: NCI-H727 cells (500 000/well) were incubated (continuous exposure, 24 h) in 6-well plates at 37 °C with increasing concentrations of 17-AAG in serum-containing medium, with levels of indicated biomarkers analyzed by western immunoblotting of equal quantities of protein from whole cell lysates. (C) Effect of increasing concentrations of the anticancer drug BMS-754807 (targeting IGF1R/IR) on NCI-H727 cell growth and constitutive IGF1R autophosphorylation, with experiments performed as described in (B). MTS data were the average \pm s.e.m. of three experiments; western immunoblotting results were representative blots from one of three experiments.

heterogeneous collection of neuroendocrine tumors, and, in particular, the high immunohistochemical levels of SSTR5, were consistent with the therapeutic importance of somatostatin analogs as inhibitory regulators of hormone hypersecretion responsible for clinical symptoms in advanced carcinoid disease. Of the five human somatostatin receptor subtypes (SSTR1–5), SSTR2 and SSTR5 have the highest binding affinity for somatostatin analogs employed clinically (primarily octreotide and lanreotide) (see review of Patil (1999)). Strong expression of somatostatin receptors on neuroendocrine tumors has long been correlated with symptomatic relief from the carcinoid syndrome in patients administered the somatostatin analog octreotide (Kvols *et al.* 1992, Modlin *et al.* 2010).

In marked contrast, KIT was not immunohistochemically expressed by the majority of neuroendocrine tumors. However, PDGFRA was moderately strongly expressed in all NETs, encouraging further study into the effect in these neoplasms of a PDGFRA-specific antibody like IMC-3G3 or anti-PDGFRA TKIs that are not dependent for activity upon the presence of activating mutations. mTOR was expressed weakly to moderately by most neuroendocrine tumors and was the only biomarker that exhibited different expression levels in primaries versus metastases (with higher expression in the former). In contrast, VEGFR1 was moderately strongly expressed in most neuroendocrine tumors, whereas Hsp90, TGFBR1, and IGF1R proteins were strongly expressed. While strong immunohistochemical staining was observed in a larger percent of BP than GI primaries for Hsp90 (74 vs 50%) and IGF1R (62 vs 44%), the high expression levels of these proteins in a large number of all neuroendocrine tumors encouraged follow-up *in vitro* studies of the effect on neuroendocrine tumor cells of anticancer drugs selective toward these biomarkers.

In experiments utilizing commercially available neuroendocrine tumor cells (three lines of BP origin), growth of the IGF1R-expressing NCI-H727 BP NET cells was inhibited by the anticancer drug BMS-754807 (an anti-IGF1R/IR TKI currently in phase I clinical trials for treatment of solid tumors) at nM concentrations which completely inhibited constitutive IGF1R phosphorylation. Proliferation of the NCI-H720 and NCI-H835 lines (with IGF1R levels undetectable by western immunoblotting) was also inhibited by BMS-754807 albeit at high (μ M) concentrations, an effect possibly due to non-IGF1R-specific activity by the dual inhibitor BMS-754807. In addition, proliferation of all three Hsp90-expressing BP NET cell lines was sensitive to 17-AAG (an Hsp90 small molecule inhibitor currently in phase III clinical trials for treatment of multiple myeloma) at nM concentrations that also induced loss of cellular EGFR and IGF1R in the EGFR- and IGF1R-expressing NCI-H727 line. These results suggested that IGF1R and Hsp90 may be molecular biomarkers important for BP neuroendocrine tumor cell growth and might serve as the basis for further research into novel targeted therapeutics for these tumors.

Scientists and clinicians attending a September 2007 summit convened by the National Cancer Institute on gastroenteropancreatic neuroendocrine tumors concurred that one reason the mean overall survival of US patients with these tumors has not changed in 30 years is the lack of data on specific molecular targets for new therapies (Modlin *et al.* 2008). Our results here

indicated that further study of new therapies for neuroendocrine tumors is warranted, e.g. presence of elevated *EGFR* copy number in 39% of all cases concomitant with infrequent occurrence of *KRAS* mutations suggests further analysis on the role of anti-EGFR monoclonal antibodies in these malignancies. In addition, strong immunohistochemical expression of ubiquitous molecular chaperone Hsp90 and of IGF1R in a majority of all neuroendocrine neoplasms is suggestive of additional research into the role that these two proteins play in neuroendocrine tumor growth; furthermore, growth inhibition of three BP NET lines by 17-AAG and of IGF1R-expressing NCI-H727 cells by BMS-754807 at concentrations of the two chemotherapeutics which induced downstream biochemical effects in (EGFR- and IGF1R-expressing) NCI-H727 cells, is encouraging of further investigation into the therapeutic possibilities of targeting IGF1R and Hsp90 for development of novel anticancer drugs for, at a minimum, bronchopulmonary neuroendocrine tumors.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-09-0318>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J A Gilbert and L J Adhikari, conception and design of study, acquisition and analysis of data, interpretation of results, drafting and critical editing of manuscript; R V Lloyd, conception and design of study, acquisition and analysis of data, interpretation of results, critical editing of manuscript; J Rubin, provision of medical perspective to study based on long-standing practice in the field of oncology, critical editing of manuscript; P Haluska, J M Carboni and M M Gottardis, provision of chemotherapeutic compound necessary for acquisition of data, critical review of manuscript based on long-standing expertise in the field of insulin-like growth factor targeting; M M Ames, conception of study, sponsoring of project, critical editing of manuscript.

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