New Medical Strategies for Midgut Carcinoids

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Abstract: Patients with well-differentiated neuroendocrine tumours of the gastrointestinal tract often present with metastases and hormonal symptoms. These patients can be palliated by interventional tumour reduction and medical treatment with somatostatin analogues; no effective chemotherapy is available. Radionuclide therapy via somatostatin receptors is one new therapeutic alternative. The recognition that neuroendocrine tumours express specific receptors for growth factors and chemokines, which are of importance for tumour growth, vascularization, and spread, may open the way for new therapeutic approaches. The signalling pathways in carcinoid tumours are incompletely explored. This review summarizes potential new treatment strategies from clinical and experimental studies, e.g. inhibition of angiogenesis, targeting of growth factors or their receptors by tyrosine kinase inhibitors, interference with specific cellular pathways (mTOR, PI3K, RAS/RAF, Notch), and also inhibition of the proteasome and histone deacetylation. Combining targeted therapy with chemotherapy, or using drugs to sensitize for radionuclide therapy, may enhance the treatment outcome.

Keywords: Angiogenesis inhibition, growth factor receptor, monoclonal antibody, multikinase inhibition, neuroendocrine tumour, radionuclide therapy, small molecule inhibitor, somatostatin receptor.

INTRODUCTION

Gastrointestinal neuroendocrine tumours can be divided into endocrine pancreatic tumours (EPTs) and carcinoids of the tubular gastrointestinal tract, of which ileal (midgut) carcinoids are the most common. Both tumour types are usually well-differentiated, with the capacity to synthesize and release biogenic amines and peptide hormones that cause specific symptoms, which can be controlled to a certain degree by long-acting somatostatin analogues.

Surgical resection is the only curative treatment of carcinoid tumours; patients with distant metastases are less often accessible for such procedures and may be subject to tumour reduction accomplished by debulking surgery, hepatic arterial embolization, or radio-frequency ablation [1]. In carefully selected patients, liver transplantation may be considered [2]. For widely disseminated disease, radionuclide therapy via highly expressed somatostatin receptors (SSTR) on the tumours, or combinations of chemotherapeutic agents or biotherapy with interferons have been attempted, but with limited success. Patients with residual metastatic disease can suffer from severe hormonal symptoms. It is therefore of some urgency to find new medical strategies to reduce tumour growth and alleviate the hormonal symptoms. Clinical studies on molecularly targeted and anti-angiogenic therapies have been started and the early results offer some promise. There have been few experimental studies on signalling pathways in midgut carcinoid tumours, and they still lack confirmation in the clinical situation.

CLINICAL THERAPEUTIC PRINCIPLES

Targeting of Somatostatin Receptors

Somatostatin receptors were first detected in the CNS and the pituitary, and were later found to be localized in the gastrointestinal tract with high expression in a large proportion of neuroendocrine tumours [3]. Somatostatin reduces smooth muscle contractility and glandular secretion by inhibiting cAMP. Reduced tumour growth was observed early in studies of cell lines [4, 5], which was attributed to hyperphosphorylation of the RB gene product and cell cycle arrest. Five subtypes of SSTR have been cloned, which differ in tissue distribution but also between tumour types, i.e. midgut carcinoids have high expression of SSTR2 and SSTR5 [6]. With the synthesis of cyclic somatostatin analogues (*octreotide*, *vapreotide*, and *lanreotide*), which are resistant to peptidases, these compounds were shown to have much longer half-lives and biological activity than native somatostatin (Fig. **1**). The analogues had high affinity to SSTR2 and SSTR5, medium affinity to SSTR3, and low affinity to SSTR1 and SSTR4 [7]. A pan-analogue (SOM 230) was developed with high affinity to all SSTRs except SSTR4 [8]. The formation of hetero-oligomers targeting dopamine- and somatostatin receptors with enhanced functional activity was also shown early on [9].

The anti-secretory effect of somatostatin analogues has been most useful to reduce carcinoid symptoms (facial flush and diarrhoea) caused by hyper-secretion of serotonin and tachykinins, and to prevent carcinoid crisis during interventional treatment [10]. Recently, an anti-proliferative effect by the depot formulation octreotide LAR was first shown in randomized studies of patients with limited tumour burden of midgut carcinoids [11]. Radiation therapy, mediated through β-emitting radionuclides bound to somatostatin analogues that are internalized into tumour cells after receptor binding, is one attractive way of treating disseminated disease [12] and may be an adjunct to intentionally curative surgery in the case of micrometastases (Fig. **1**). Tailored chemotherapy for several tumour types based on their expression of peptide receptors is currently under investigation [13].

Targeting of EGFR and PDGFR

Therapy targeting specific growth factors and their receptors, e.g. inhibition of epithelial growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) pathways in advanced colon cancer, or of the EGFR tyrosine kinase in non-small cell lung carcinoma (NSCLC) after failure with chemotherapy, has encouraged the use of similar principles for neuroendocrine tumours. These tumours can express both receptors for platelet-derived growth factor (PDGF) and VEGF and their ligands as part of autocrine/paracrine loops [14, 15]. In immunohistochemical studies, VEGF was found to be present in half of the EPTs studied; and EGFR, the tyrosine kinase receptor KIT (with stem cell factor as ligand), and basic fibroblast growth factor (bFGF) were present in about one quarter. The pattern differed from that of carcinoids, which almost invariably expressed EGFR, and VEGF also in more than half of the tumours [16]. EGFR expression was frequently upregulated and it was suggested to influence the growth and spread of these tumours [17]. PDGFA and PDGFB may be expressed in both tumour and stromal tissue [14].

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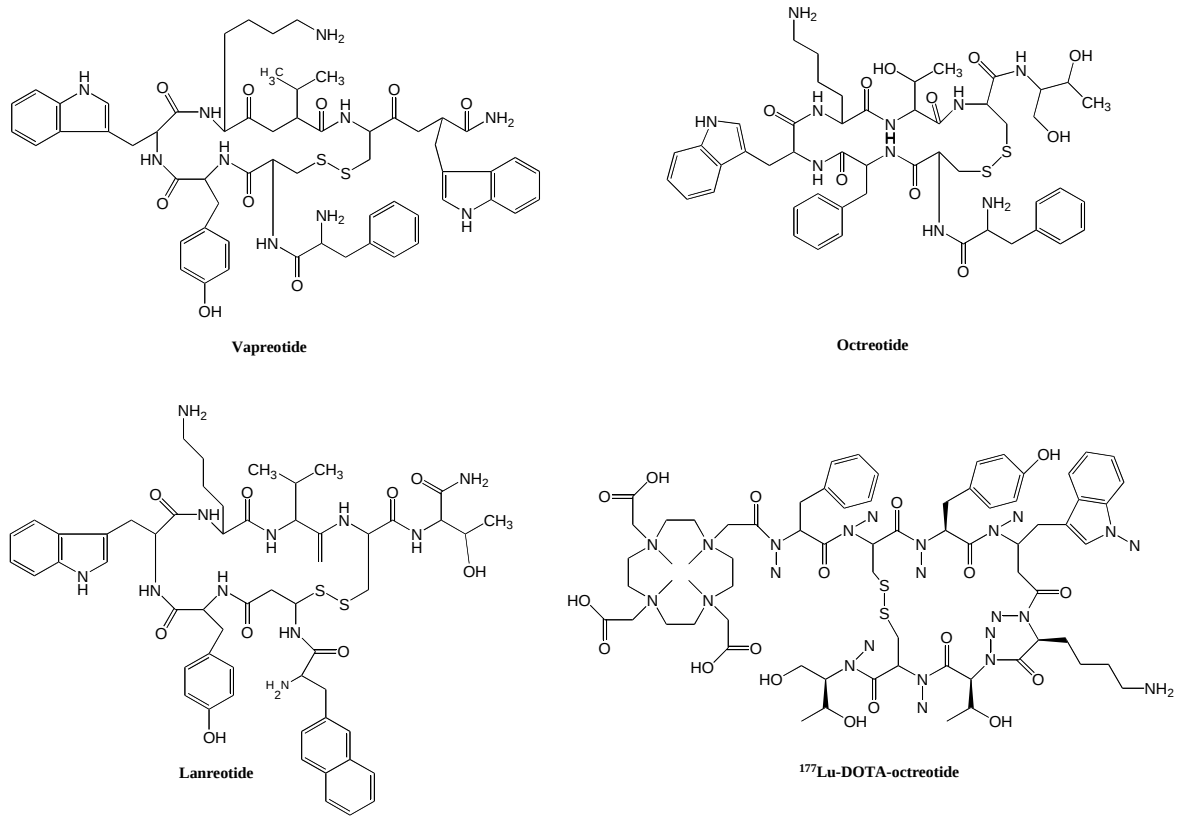

Fig. (1). Three commonly used somatostatin analogues. Radiolabelled octreotate is used for radionuclide therapy.

The first clinical studies targeting tyrosine kinases were performed in almost 100 patients with progressive disease after treatment using the EGFR tyrosine kinase inhibitor (TKI) *gefitinib* [18] (Fig. **2**). Radiological tumour regression was observed in less than 10% of cases, but tumour stabilization was more frequent; it was notably more common in patients with carcinoids than in patients with EPT (the 6-month progression-free survival was 30% *vs*. 10%). In an ongoing trial of advanced gastrointestinal cancer, including carcinoids, another EGFR-TKI, *erlotinib*, was combined with the EGFR-targeted antibody cetuximab (to tag tumour cells with IgG for the immune effector cells) (NIH: NCT 00397384). In studies of other tumour types, EGFR targeting appeared to have optimal effects first when combined with other targeted therapies or chemotherapy [19-21]. Hobday and collaborators[1] studied a multi-targeted TKI, *sorafenib* (VEGFR2-3, PDGFR, KIT, the Fms-Like Tyrosine kinase 3 (FLT3), the oncoprotein BRAF, and FGFR1) leading to radiological RECIST (Response Evaluation Criteria in Solid Tumours) responses in about 10% of the patients, which were equally frequent in carcinoids and EPT. Due to the complex actions of this drug, it can be difficult to ascribe the observed effects to interference with the oncogenic pathway RAF/MEK/ERK, anti-angiogenic activity, or other mechanisms. It must be borne in mind that when signalling pathways are inhibited in tumour cells, alternative pathways can be activated, leading to reduced therapeutic effects [22]. The spontaneous growth of neuroendocrine tumours may vary considerably, which means that disease stabilization can be difficult to evaluate if data on tumour progression prior to therapy are not available. Another problem with phase-II studies is that recruited patients can have considerable selection bias due to numerous previous treatments.

Sunitinib is a multi-target TKI (VEGFR1-3, PDGFR, KIT, the oncoprotein RET, and FLT3), which was also studied in almost 100 patients with progressive neuroendocrine tumour disease (Fig. **2**). Also here, tumour regression by RECIST criteria was unusual (16% for EPT but only 2% for carcinoids), while tumour stabilization was much more frequent (75–93%) [23]. A phase-III trial in patients with advanced EPT was recently stopped due to differences in efficacy; the sunitinib-treated group had a progression-free survival of 11.1 months as opposed to 5.5 months in the placebo group[2]. Preliminary results on sunitinib combined with hepatic arterial embolization are also encouraging[3].

Imatinib, the first TKI tailored for treatment of chronic myeloic leukemia and gastrointestinal stromal tumours (GIST), also targets PDGFR, which may be expressed by carcinoids. This drug has weak effects on PDGFR in comparison with sunitinib and has no effect on VEGFR (Fig. **2**). Accordingly, in limited studies of advanced carcinoids only one patient showed objective responses; drug-related toxicity was seen due to high dosage [24, 25]. It can still not be excluded that imatinib has a role as an adjunct to VEGF inhibition [26]. For EPT and carcinoids, specific activating mutations of target kinases cannot be related to therapeutic outcome, i.e. a success similar to that of imatinib in GIST should not be expected. With knowledge of the mutational status of critical target

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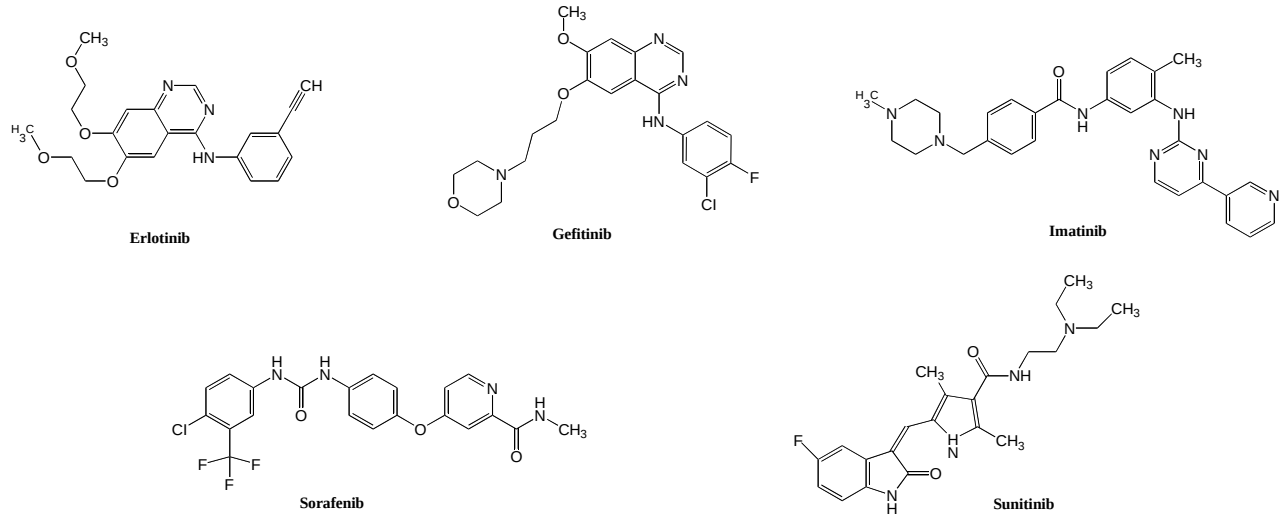

Fig. (2). Small-molecule inhibitors of EGFR (gefitinib, erlotinib) and multi-target TKIs (sorafenib, sunitinib). Imatinib was the first TKI to target KIT and PDGFRA.

genes in GIST, the choice of an optimal drug can be made, e.g. imatinib for patients with *KIT* exon 11 mutations, sunitinib for *KIT* exon 9 mutations, or in the case of drug resistance due to acquired tyrosine kinase mutations a multi-target TKI can be used [27]. Similar principles can be used to some extent for other tumour types, e.g. NSCLC. The demonstration of mutational activation of *EGFR* in these tumours is helpful in selecting patients who will respond to gefitinib, while a high number of *EGFR* gene copies characterizes patients who are responsive to erlotinib [28].

Targeting of mTOR

The protein kinase mammalian target of rapamycin (mTOR) is a downstream regulator of signalling from several growth factors, e.g. via the phosphatidylinositol 3 kinase (PI3K)/AKT pathway, and influences the early protein translation that is a prerequisite for cell cycle progression. Under normal conditions, PI3K/AKT is controlled by a suppressor gene, phosphatase and tensin homologue (*PTEN*). When *PTEN* is inactivated by mutations, epigenetic events, or altered subcellular localization of its protein, mTOR is activated and can participate in the tumorigenesis [29, 30]. It is therefore of interest that phosphorylation of AKT has been observed in neuroendocrine tumours and that inhibition of mTOR induces anti-proliferative effects *in vitro* [29, 31]. Modern mTOR inhibitors (*everolimus* and *temsirolimus)* are analogues of the natural antibiotic rapamycin (*sirolimus*) that was originally used for immunosuppressive treatment in patients undergoing organ transplants (Fig. **3**). They have been used with some success as rescue therapy in patients with GIST and acquired TKI-resistance [27]. In a limited study, *everolimus* was combined with octreotide LAR (to reduce autocrine/paracrine secretion of growth factors) in patients with EPT or carcinoids. Chromogranin A (CgA) is a granular neurosecretory protein released together with tumour-specific hormones; it serves as a general plasma marker of neuroendocrine tumour disease. After treatment with everolimus, the CgA levels were reduced and long, progression-free intervals were seen; the 6-month progression-free survival was 64%. Again, EPT appeared to be more responsive than carcinoids (27% as opposed to 17%) and the treatment was well tolerated [32]. The confirmatory phase-II trials in progressive refractory EPT have recently been reported (RADIANT 1) [33]. The RADIANT 2 study is directed at advanced carcinoids.

Inhibition of Angiogenesis

Antiangiogenic therapy has recently been attempted in phase-II studies of patients with neuroendocrine tumours, since these tumours are often well vascularized and frequently express VEGF (or its subtypes VEGF B-E) and/or the placental growth factors (PlGF1 and PlGF2) [15, 34]. Furthermore, there is a correlation between circulating levels of VEGF and progression of these tumours [35] and also between elevated expression of VEGF and both enhanced angiogenesis and reduced progression-free survival [36]. Also, other angiogenic factors, e.g. bFGF and PDGF sub-types, can be released from tumour cells or tumour vasculature. When VEGF binds to high-affinity receptors on the endothelium (VEGFR1 and VEGFR2), or on lymph vessels (VEGFR3), the intracellular domain of the receptor becomes phosphorylated. This in turn activates downstream signalling, leading to angiogenesis. Inhibition of this process can be accomplished with antibodies to VEGF or its receptors, or with TKI, which inhibits receptor phosphorylation. The second generation of TKIs after imatinib (*sunitinib* and *sorafenib*) target several tyrosine kinases, including those of VEGFR1–VEGFR3, and they therefore inhibit angiogenesis also. Recombinant endostatin mimics the endogenous anti-angiogenesis, while metalloproteinase inhibitors influence the degradation of the extracellular matrix, which is an early step in angiogenesis.

Bevacizumab (a monoclonal antibody to VEGF) has a proven therapeutic effect when combined with chemotherapy in colon cancer [37]. In a phase-II trial, bevacizumab was tested as monotherapy vs. pegylated interferon-α for advanced carcinoids; bevacizumab led to improved progression-free survival and markedly reduced tumour perfusion[4]. When octreotide was combined with bevacizumab, patients with carcinoids more frequently demonstrated early tumour remissions than after treatment with pegylated interferon-α and octreotide [38]. When bevacizumab was combined with the alkylating agent *temozolomide* (a dacarbazine analogue), partial remissions and stable disease were seen more often in patients with EPT than in patients with carcinoids, which may reflect differences in vascularization between the tumour types[5]. When bevacizumab was combined with standard treatment of gastrointestinal cancer in phase-II trials of patients with neuroendocrine tu-

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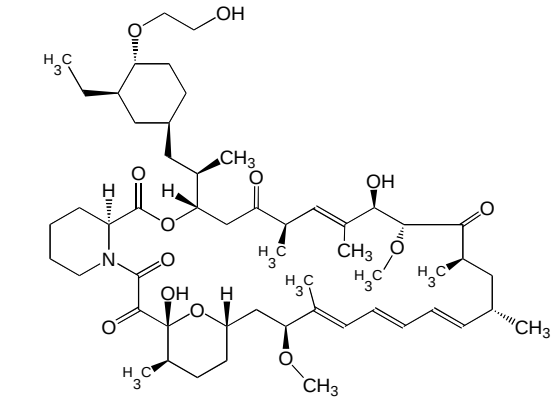
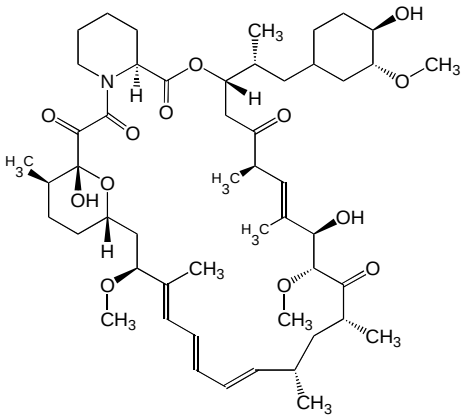
mours, e.g. capecitabine and oxaliplatin or FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin), 20–30% of the patients showed partial responses[6,7]. Bevacizumab has also been investigated in patients with advanced carcinoids in combination with 2-methoxyestradiol (NIH: NCT00328497). The latter substance can reduce growth and induce apoptosis in both endothelium and tumour cells, and inhibit hypoxia-inducible factor (HIF-1), which drives pro-angiogenic genes [39, 40].



Everolimus



Sirolimus

Fig. (3). Inhibitors of mTOR.

Vatalanib inhibits all VEGFR tyrosine kinases and might therefore block both tumour angiogenesis and lymphangiogenesis (Fig. **4**). Relatively few patients with neuroendocrine tumours have been studied in phase-I and phase-II studies, demonstrating no radiological responses so far, but with a high frequency of disease stabilization[8,9]. Common side effects (nausea and vomiting) were mild, but hypertension occurred in individual patients.

Two pan-VEGFR inhibitors, *pazopanib* and *AMG706*, have been developed with additional inhibition of PDGFR and KIT [41, 42] (Fig. **4**). Monotherapy with AMG706 is presently tested in patients with low-grade neuroendocrine tumours to assess tolerability and early anti-tumour effects (NIH:NCT00427349), while pazopanib has reached phase-II studies (NIH:NCT00454363).

In animal studies, anti-PlGF monoclonal antibodies were shown to block the angiogenic switch in tumours without influencing the physiological vasculature and may be useful in cases of resistance to VEGF inhibitors [43]. The antibodies also appear to block rescue-angiogenesis, which can be a problem in long-term anti-angiogenic therapy.

Thalidomide interferes not only with VEGF and bFGF signalling, but also with the formation of extracellular matrix. Thalidomide is therefore a multifaceted anti-angiogenic drug. Together with *temozolomide*, it has been used in a phase-II trial in 29 patients with neuroendocrine tumours. The overall radiological response rate was 25% and tumour stabilization was almost three times higher, but at the cost of high toxicity (fatigue, thrombocytopenia, neuropathy) [44] (Fig. **4**).

Recombinant endostatin (a 20-kD fragment of collagen XVIII) was tested in a phase-II trial of 42 patients with neuroendocrine tumours. No radiological responses were observed, but 80% showed stable disease for a median of 11 months and the toxicity was low [45].

Anti-angiogenic therapy is thus a new approach of some promise in neuroendocrine oncology. In the studies cited, partial tumour responses were unusual in patients with carcinoids (< 10%), but appeared to be about twice as common in patients with EPT.

EXPERIMENTAL THERAPEUTIC PRINCIPLES

The study of neuroendocrine tumours has been hampered by a number of circumstances, which were recently reviewed [46]. The main obstacles to progress in gastroenteropancreatic neuroendocrine tumour management include (1) our limited understanding of the cellular and molecular biology of neuroendocrine cells and the mechanisms of tumorigenesis, (2) a shortage of *in vitro* and animal models to study disease pathogenesis and treatment, (3) a paucity of critical targets for new therapies, and (4) a lack of uniform pathological classification and staging systems. The lack of a uniform classification system is evident in most clinical studies on neuroendocrine tumours. The carcinoid tumour group is heterogeneous and comprises a mixture of foregut-, midgut-, and hindgut-derived tumours. The clinical response rate of carcinoid tumours is often compared to that of EPTs, which are also a heterogeneous group of tumours with marked differences in clinical presentation and malignant behaviour. Furthermore, clinical studies are commonly performed on patients with tumours displaying varying degrees of differentiation, proliferation rates, and disease stage, thus limiting the validity of the conclusions drawn. Experimental data from well-characterized *in vitro* and animal models are therefore needed to properly evaluate novel treatment principles for ileal carcinoids. To date, five human carcinoid cell lines have been characterized: the pancreatic carcinoid cell line BON [47] and the ileal (midgut) carcinoid cell lines KRJ-1 [48], GOT1 [49], CNDT2 [50], and STS [51]. Three of the cell lines (BON, KRJ-1, and GOT1) have been used to identify therapeutic targets; only two models, KRJ-1 and GOT1, are representative of enterochromaffin cell (ileal) carcinoids [52, 53]. In the following sections, we give different examples of anti-tumour effects demonstrated in carcinoid cells *in vitro* and in animal models; these effects suggest possible new treatment principles for ileal carcinoids.

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[8] Anthony, L.; Chester, M.; Michael, S.; O'Dorisio, T. M.; O'Dorisio, M. S. Phase II open-label clinical trial of vatalanib (PTK/ZK) in patients with progressive neuroendocrine cancer. *J. Clin. Oncol. (Meeting Abstracts)*, **2008,** *26*(15 suppl), 14624.

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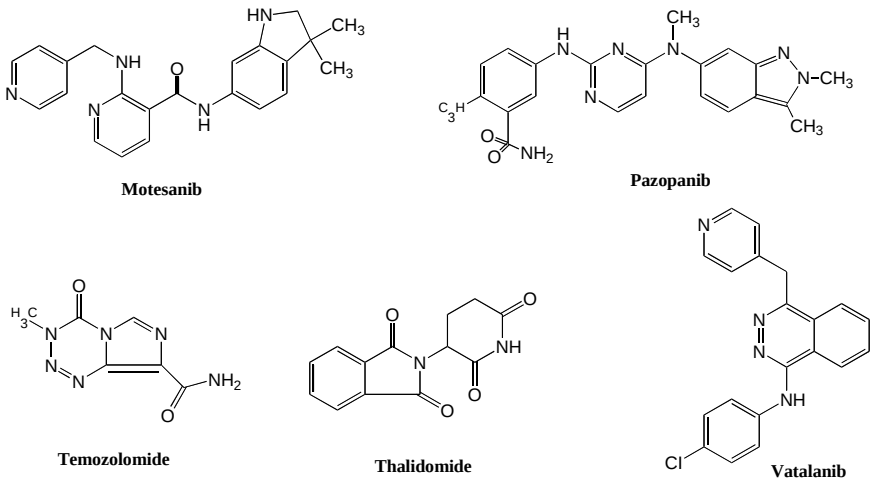


Fig. (4). Inhibitors of angiogenesis; motesanib, pazopanib, and vatalanib are TKIs.

Targeting of PARP

Nuclear factor-κB (NF-κB)/Rel transcription factors are important regulators of both inflammation and tumorigenesis. The tumorigenic effects of activated NF-κB include promotion of malignant transformation, proliferation, inhibition of apoptosis, invasiveness, angiogenesis, and metastasis formation [54]. Several human tumour types have been shown to harbour constitutively activated NF-κB [55-60] and in some cases mutations associated with NF-κB signalling have been found [61]. The most effective way of interfering with NF-κB activation is to inhibit the activity of the IκB (inhibitory protein of NF-κB) kinase (IKK) [62] (Fig. **5**). *CHS828* (or *GMX1778*) is a pyridyl guanidine with anti-tumour activity *in vitro* and *in vivo*. Earlier studies have shown that CHS828 inhibits the activation of NF-κB in several tumour cell lines and that the degree of tumour reduction is related to the inhibition of IKK (Fig. **6**). It was suggested that the drug directly inhibited phosphorylation of IκB and thus the activation of NF-κB [63]. The survival of a neuroendocrine tumour cell line (TT: medullary thyroid carcinoma) and the tumour reduction of xenografted SCLC (NYH: small cell lung cancer) was found to be critically related to the activity of IKK [63, 64]. CHS828 was demonstrated to have potent anti-tumour effects also on xenografted neuroblastoma [65]. The concentration of CHS828 that caused apoptosis *in vitro* (1–10 nM) was in the same range as the IC_{50} for IKK activity [63].

In our own studies, we have demonstrated a strong anti-tumour effect of CHS828, both *in vitro* and *in vivo*, on cell lines of three different neuroendocrine tumour types: ileal carcinoid (GOT1), medullary thyroid carcinoma (GOT2), and pancreatic carcinoid (BON). When xenotransplanted to nude mice, one weekly oral dose of CHS828 led to necrosis and complete tumour regression of both GOT1 and GOT2 within 2–3 weeks (250 mg/kg); a complete regression of BON tumours was also achieved, with no observed adverse effects [66] (Fig. **7**).

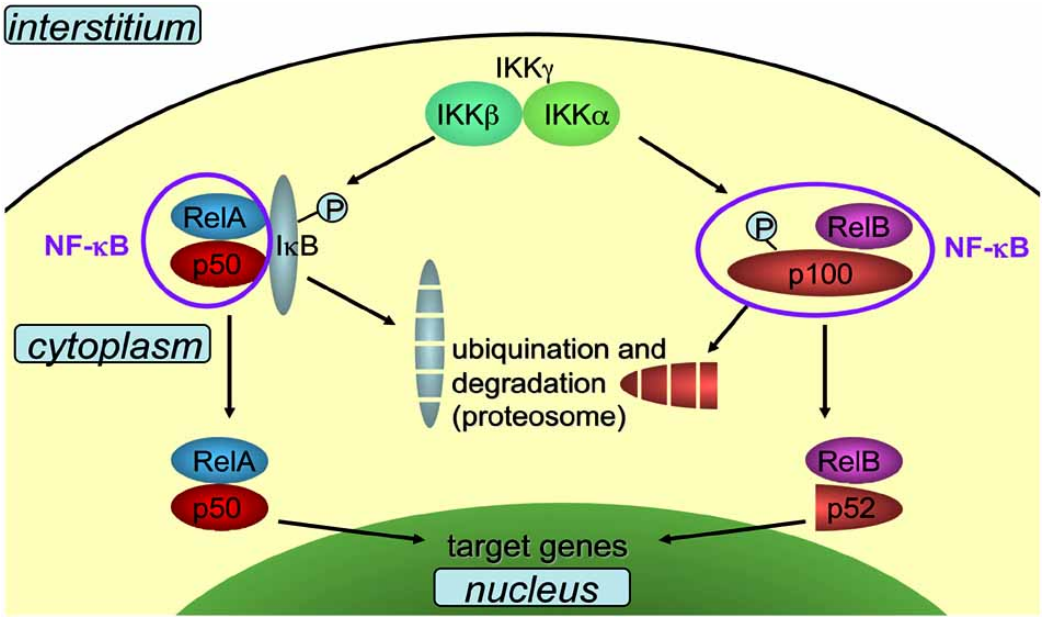
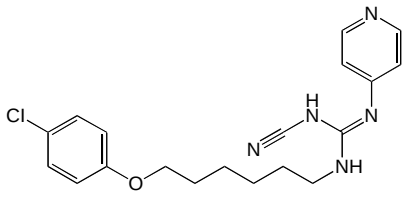
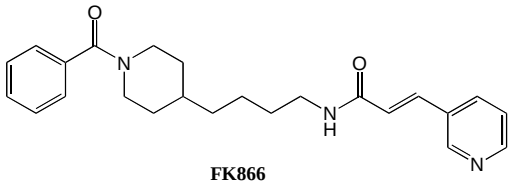


Fig. (5). Nuclear factor-κB signalling and interaction with the proteasome.
Nuclear factor-κB (NF-κB) is a group of transcription factors that mediate anti-apoptotic and pro-survival signals. NF-κB is constitutively activated in many tumour types. Inactive NF-κB is constrained in the cytoplasm by a binding protein, IκB (inhibitor of NF-κB). Upon stimulation, IKK (IκB kinase) phosphorylates IκB. This leads to ubiquitin-dependent degradation of IkB and release of the NF-κB heterodimer (i.e. RelA/p50), allowing it to translocate to the nucleus.



CHS828



FK866

Fig. (6). Inhibitors of PARP and NAMPT.

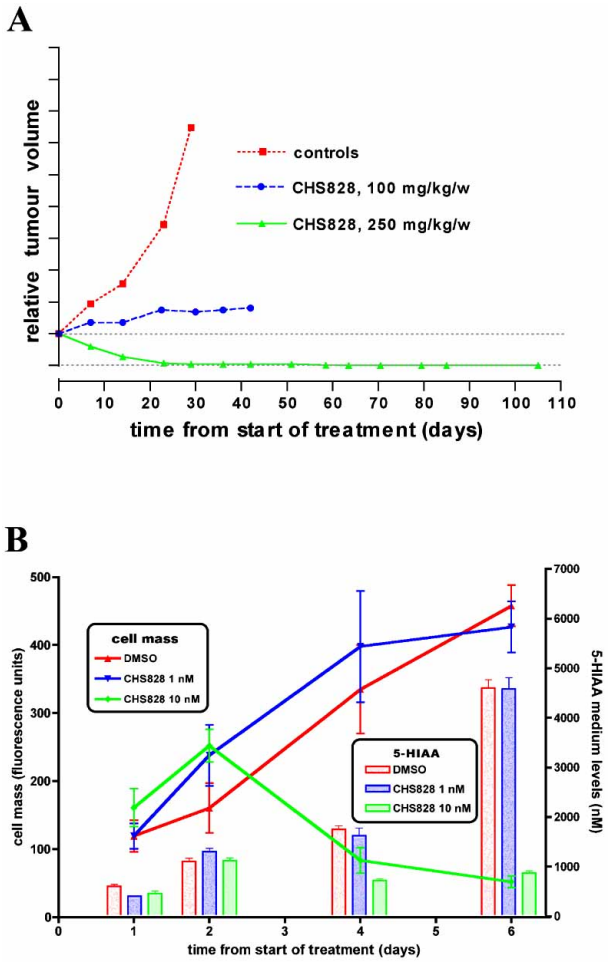


Fig. (7). Anti-tumour effects of CHS828.
(A) CHS828 was administered to nude mice xenografted with human midgut carcinoid (GOT1) once weekly by oral gavage: 100 mg/kg (days 0–42) or 250 mg/kg (days 0–57). The higher dose resulted in total tumour regression. **(B)** CHS828 was administered to the midgut carcinoid cell line GOT1 *in vitro* (0 nM (control), 1 nM, or 10 nM for 3 days). There was pronounced inhibition of cell growth at the higher dose, parallelled by depletion of intracellular serotonin.

An alternative mechanism of action for CHS828 involves NAD^+ metabolism [67]. Nicotinamide adenine dinucleotide (NAD^+) is an essential co-enzyme in cellular redox reactions, e.g. generation of ATP, but is also an important substrate in ADP ribosylation. Poly(ADP-ribose) polymerase 1 (PARP1) is an evolutionarily well-conserved nuclear enzyme with the key function of signalling genotoxic stress/DNA repair. The PARP family of enzymes includes 18 members, of which PARP1 and PARP2 are the only ones activated by DNA damage. Upon binding to DNA strand breaks, PARP1 metabolizes NAD^+ into branched polymers of ADP-ribose, which are transferred to nuclear proteins including PARP1 itself [68, 69]. Poly(ADP-ribos)ylation has been proposed to function in genome repair by facilitating the opening of the condensed structure of chromatin required for the recruitment of the repairing enzymes. PARP1 activation thus consumes NAD^+, which is cleaved to nicotinamide and ADP-ribose. NAD^+ can be resynthesized via the so-called "salvage pathway". Thus, nicotinamide is first converted to nicotinamide mononucleotide (NMN) by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) and then to NAD^+ by nicotinamide mononucleotide adenyltransferase (NMNAT) [70]. Overactivation of PARP1 by massive DNA damage, e.g. radiotherapy, leads to depletion of NAD^+/ATP energy stores and results in cellular death [71]. Tumour cells have high energy demands and PARP1 is constitutively activated due to DNA damage, which is the background to the new principle of cancer treatment by inhibition of NAD^+ synthesis [72]. Olesen and collaborators found that CHS828 reduced the intracellular levels of NAD^+ and impaired its re-synthesis to a similar degree to a structurally unrelated NAMPT inhibitor, *FK866*, and also demonstrated cross-resistance with this drug [67] (Fig. **6**). CHS828 and FK866 were thus suggested to inhibit the NAD+ salvage pathway [67] (Fig. **8**). Watson and collaborators have recently confirmed that the mechanism of action of CHS828 is through potent and specific inhibition of NAMPT [73]. *In vitro* studies revealed that both NAD+ and ATP were depleted by treatment with CHS828, but the NAD^+ depletion preceeded that of ATP. Furthermore, nicotinic acid protected against the cytotoxicity caused by CHS828 (presumably via the Preiss-Handler pathway, an alternative salvage pathway of NAD^+ regeneration) (Fig. **8**). The results indicated that the observed inhibition of IKK and NF-κB activation upon exposure to CHS828 is secondary to the NAD^+ depletion. Since GOT1 cells are sensitive to CHS828 both *in vitro* and as xenografts, the effects of CHS828 have to be compared with that of other NAMPT inhibitors in the same experimental models (Fig. **7**).

At present, 104 patients in total have received FK866, or CHS828, as monotherapy in phase-I trials [74]. Adverse effects with thrombocytopenia and gastrointestinal toxicity were significant and no tumour regression was seen in any of the five published studies. It has therefore been suggested that if NAD^+-depleting drugs are to be used clinically, it will probably be in combination with therapies causing DNA damage and NAD^+ depletion [74].

Targeting of the Proteasome

The 26S proteasome complex degrades proteins tagged with ubiquitin, and this mechanism is referred to as the ubiquitin-proteasome pathway (UPP). This non-lysosomal proteolytic system thus destroys proteins that are of importance for the cell cycle, apoptosis, and NF-kB activation (Fig. **5**), as well as mutant proteins [75]. Inhibition of UPP is therefore an interesting target for cancer therapy. *Bortezomib* inhibits active threonine sites of the proteasome and has been tested on neuroendocrine tumour cells [76] (Fig. **9**). In one small study of patients with carcinoids and EPT, disease stabilization was seen; neuropathy occurred in one third [77]. In studies on neuroendocrine tumour cells, the combination of histone deacetylase (HDAC) inhibition (see below) and bortezomib had marked anti-proliferative activity and induced apoptosis [78].

Targeted Radionuclide Therapy

It is generally assumed that carcinoid tumours are relatively radioresistant, and that external radiation therapy is only indicated for palliation or for regionally advanced or metastatic disease [79-

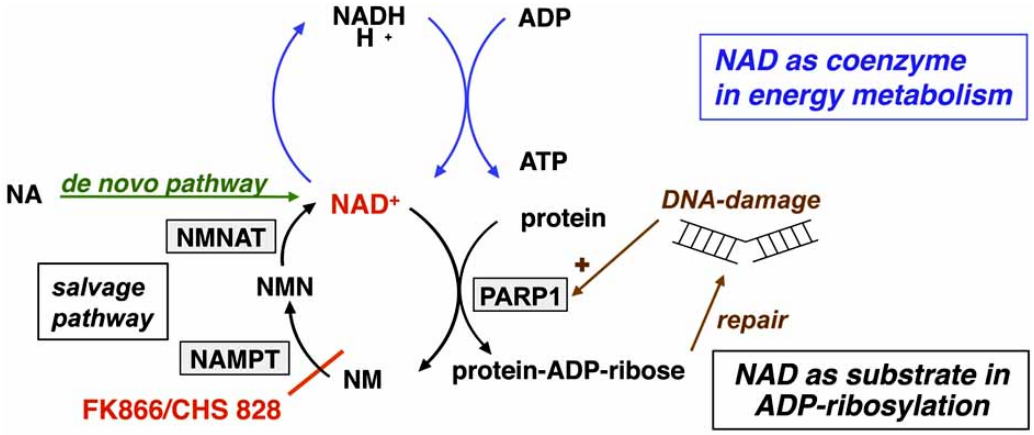
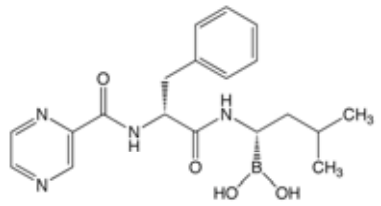


Fig. (8). Interaction between NAD^+ and ATP production

NAD^+ is essential for enzymatic redox reactions involved in cellular metabolism, including ATP production. It is also a substrate of poly(ADP) ribosylation, a reaction catalyzed by PARP1 (poly(ADP-ribose) polymerase), which is activated by DNA strand breaks. This reaction consumes NAD^+, which is cleaved to nicotinamide (NM) and ADP-ribose. NAD^+ can be re-synthesized either from exogenously administered nicotinic acid (niacin, NA) or from NM via the salvage pathway. Here, NM is first converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT) and then to NAD^+ by nicotinamide mononucleotide adenyltransferase (NMNAT). CHS828 and FK866 inhibit the NAD^+ salvage pathway as indicated.

84]. The assumption has persisted, although published studies on the clinical effects of external radiation therapy have actually revealed partial or complete remission in 25-80% of the patients after a radiation dose of 20–50 Gy to the tumour tissue [85-88].



Bortezomib

Fig. (9). Inhibitor of the proteasome.

Despite the assumed radioresistance of carcinoid tumours, SSTR-mediated radiation therapy has been introduced for neuroendocrine tumours, showing both promising effects and moderate toxicity [12, 89]. We have presented successful therapeutic results of ^{177}Lu-[DOTA^0, Tyr^3]-octreotate (*^{177}Lu-octreotate*) in nude mice carrying GOT1 tumours [90] (Fig. **1**). After intravenous injection of ^{177}Lu-octreotate, a rapid dose-dependent tumour regression was obtained (Fig. **10**). Complete remission (>99% reduction of tumour volume) was obtained in 33% of the mice at 15 MBq, and in 100% at 30 MBq—corresponding to an absorbed dose of 60 and 120 Gy, respectively. Increased apoptosis was found one and three days after administration. Already 7 days after injection, the number of tumour cells was significantly reduced, and the necrosis and oedema demonstrated at day 3 were now replaced by fibrosis (Fig. **10**).

We have previously found increased uptake of ^{177}Lu-octreotate in SCLC cells after irradiation *in vitro*, probably due to up-regulation of SSTR expression as demonstrated by increased SSTR2 mRNA expression [91]. These results were verified *in vivo* in nude mice xenografted with GOT1 [92]. After low amounts of ^{177}Lu-octreotate, a twofold higher concentration of subsequently given ^{111}In-octreotide was obtained in the tumour tissue. These results may be useful in optimizing therapy using radiolabelled somatostatin analogues. By choosing optimal time schedule and dosage, enhanced therapeutic effects might be obtained without increased side effects.

Apoptotic Pathway

Ionizing radiation induces DNA damage, which is followed by a number of cellular responses, including DNA repair, cell cycle arrest, autophagy, mitotic catastrophy, necrosis, and apoptosis [93, 94]. Our understanding of the molecular mechanisms underlying the response to DNA damage is limited. Recent studies indicate that radiation-induced cell death can be divided into several distinct pathways according to the time course and the position in the cell-cycle; the apoptotic response plays a key role in almost every mode of cell death [94]. The tendency to undergo apoptosis varies between different cell types. Pre-mitotic apoptosis is rapid and is associated with a prompt activation of caspase 3, a key enzyme of intracellular signalling of apoptosis, while post-mitotic apoptosis is delayed until after cell division, and does not require rapid activation of caspase 3, but is associated with down-regulation of anti-apoptotic genes such as *MAPK* and *BCL2* [94]. Furthermore, radiation induces apoptosis both via the intrinsic mitochondrion-mediated and extrinsic death-receptor-mediated pathways. Apoptosis can thus be initiated in different cell compartments, e.g. the nucleus, the cytoplasm, or the plasma membrane [93]. There is evidence to suggest that radiation may induce biological responses via extranuclear targets and extracellular events [95-98].

Radiation-induced effects may vary in different types of tumour cells, but also between different types of normal cells due to the status of the signalling pathways in the cell. We have clearly demonstrated that irradiation by ^{177}Lu-octreotate induces apoptosis in GOT1 tumours in nude mice with a maximum at day 1 and 3 after administration of 30 MBq ^{177}Lu-octreotate [90] (Fig. **10**).

Bystander Effect

Radiation-induced bystander effects have been demonstrated for a variety of biological endpoints in both human and animal cell lines, whereby non-irradiated cells are affected by irradiated cells. The mechanisms behind this phenomenon are incompletely known [95, 96]. Mechanisms that have been suggested are communication via gap junctions, Ca^{++} channels, cytokine or growth factor receptors, and small soluble mediators (reactive oxygen species (ROS), e.g. NO). The various signal transduction pathways involve the MAPK superfamily, i.e. ERK 1/2, c-Jun N-terminal kinase (JNK), and p38 kinase. Other mechanisms include the cyclooxygenase-2 (COX-2) signalling cascade, which can modulate cellular inflammation and genomic instability, IGF signalling pathways via IGFBP-3, and NAD(P)H oxidase, leading to long-lasting ROS pro-

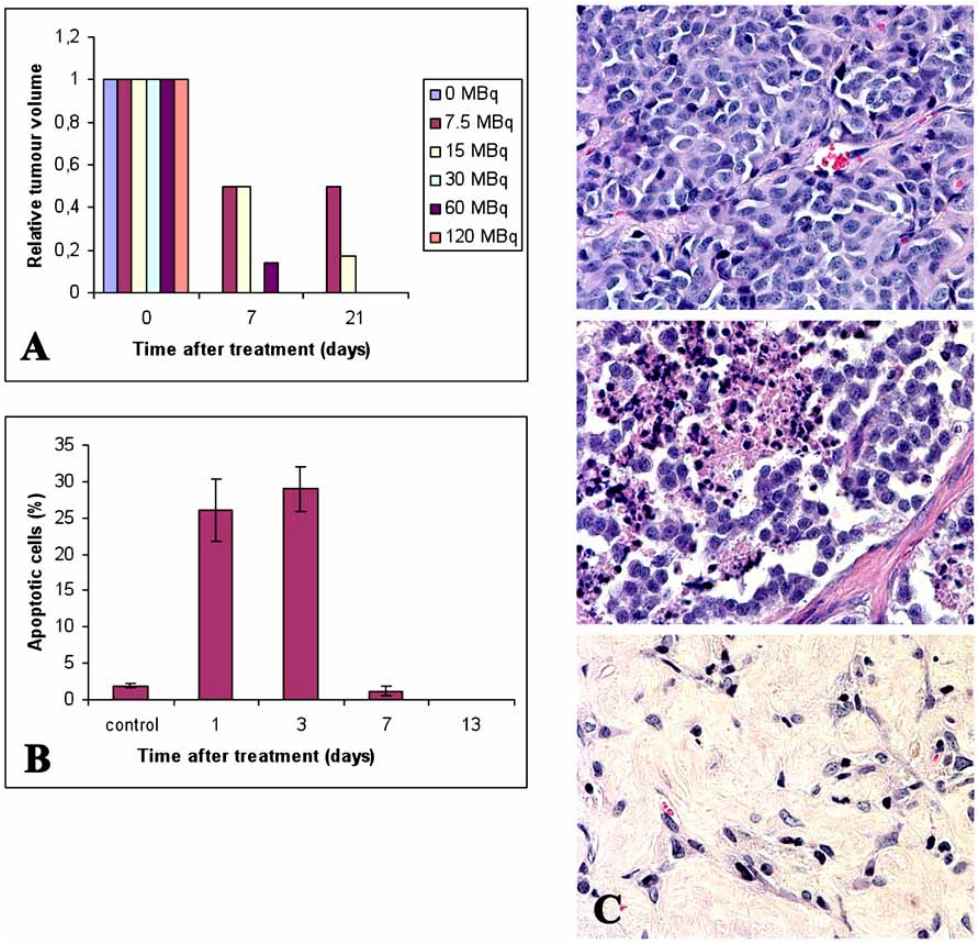


Fig. (10). Anti-tumour effects of ^{177}Lu-octreotate

(A) ^{177}Lu-octreotate was administered to nude mice xenografted with human midgut carcinoid (GOT1); 7.5–120 MBq, resulted in dose-related tumour regression. (B) Relative number of apoptotic cells 1–13 days after treatment. (C) Micrographs of GOT1 cell xenografts in nude mice 0 (top), 7 (middle), and 13 days (bottom) after administration of 30 MBq ^{177}Lu-octreotate.

duction [96]. The *COX-2* gene is expressed in many carcinoids, and high expression of its protein in primary midgut carcinoids may be associated with a negative prognosis [99].

The importance of bystander effects in radiation therapy has been discussed, and they are potentially beneficial, especially in radionuclide therapy where more tumour cells could be affected than those targeted. The unexpectedly rapid GOT1 tumour cell reduction in our experimental studies might reflect a bystander effect (Fig. **10**).

Interaction between Radiation and the Apoptotic Pathway

Anti-tumour agents that restore apoptosis signalling in cancer cells may induce tumour regression and improve conventional treatment such as radiation therapy. To enhance therapeutic effects, there is probably a need to combine radiation with one or several pro-apoptotic or anti-survival stimuli. In many tumours, including carcinoids, the apoptotic signalling is disrupted or reduced [58]. The relative radio-resistance might be due to such a resistance to apoptosis. One important way to enhance the cytotoxic effect of radiation therapy is by modulation of the apoptotic response (inhibition of anti-apoptotic signals) selectively in tumour cells by interventions involving specific signal transduction pathways (radiosensitization).

There are several strategies to increase apoptotic signalling or reduce anti-apoptotic signalling in tumour cells, e.g. agonistic monoclonal antibodies specific for death receptors, monoclonal antibodies competing with growth receptors, TKIs, small-molecule inhibitors to block e.g. Bcl-2 proteins, anti-sense oligonucleotides, or siRNA [100]. If such techniques are used, they should be active only on cancer cells without having any effect on normal cells. In many tumour types, several strategies have been suggested and tested both experimentally and in pilot clinical trials [100].

Carcinoids express *BCL2* and *MYC* to a high degree, while *p53* is seldom over-expressed or mutated [101, 102]. High levels of Bcl-2 protein expression may thus be linked to the intrinsic resistance to chemotherapy and radiation therapy [101]. Bcl-2 inhibits apoptosis, and inhibition of this protein might stimulate apoptosis and increase the radio-sensitivity of carcinoids. As mentioned above, nude mice xenografted subcutaneously with GOT1 tumours were found to be curable with high doses of ^{177}Lu-octreotate or CHS828 (30 MBq intravenously and 250 mg/kg/week orally, respectively) [66, 90]. In a recent experimental study on xenografted nude mice, we studied CHS828 as a radio-sensitizer. Much lower doses of ^{177}Lu-octreotate or CHS828 (7.5 MBq and 100 mg/kg/week, respectively) resulted in partial regression of tumours, while the combination of the two therapies resulted in complete tumour regression in almost all the animals (Fig. **11**). As CHS828 inhibits the regeneration of NAD+ and since there is a synergistic effect of CHS828 and radiation therapy, PARP1 activation may be involved in the death mechanism induced by radiation under these conditions.

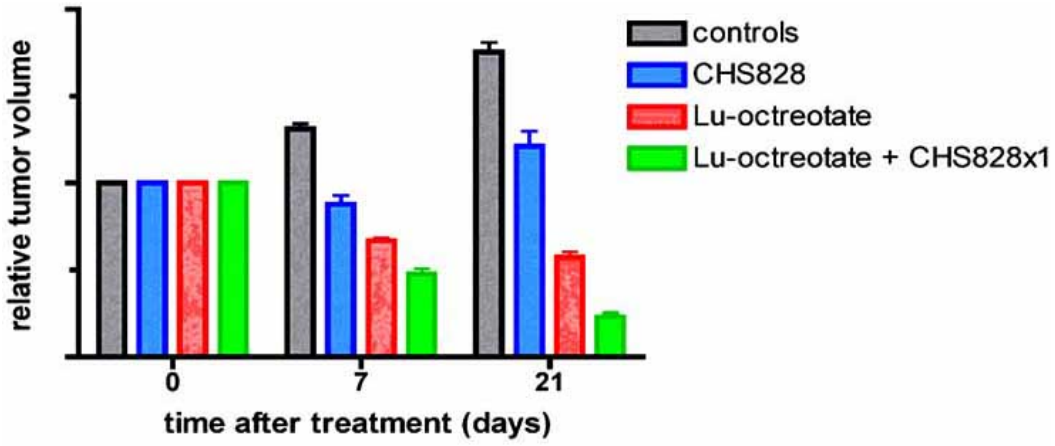
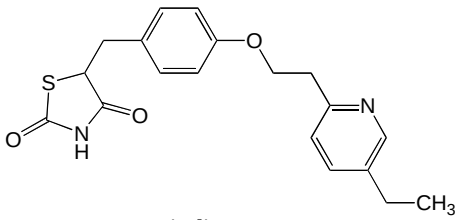


Fig. (11). Radio-sensitizing effects of CHS828 on carcinoid tumour cells
Nude mice xenografted with human midgut carcinoid GOT1 were treated with ^{177}Lu-octreotate (7.5 MBq intraperitoneally) with a single dose of CHS828 (100 mg/kg per os) or with a combination with these two treatments; the combined treatment resulted in enhanced therapeutic effect.

Death Receptor Signalling

The death receptors belong to the tumour necrosis factor (TNF) receptor superfamily, which is characterized by cysteine-rich extracellular domains and a cytoplasmic death domain that serves as the recognition point for the apoptotic machinery. Much interest has been focused on death receptor signalling, especially regarding TRAILR1 and TRAILR2. TRAIL, the natural ligand of TRAILR1 and -2, preferentially kills tumour cells but has no effect on normal cells. However, TRAIL has a short half-life *in vivo* [103-106], which makes it a less attractive alternative for therapy. Instead, agonistic TRAILR antibodies and recombinant ligands have been developed. TRAIL resistance may also be due to increased expression of anti-apoptotic proteins, e.g. Bcl-2 and c-FLIP [107]. In gastrointestinal tumours, specific TRAIL-induced signalling can be obtained from proteasome or HDAC inhibitors with no response in normal cells [100, 108, 109] (see below). TRAIL-induced apoptosis has been studied in carcinoid cells *in vitro*, and a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist *pioglitazone* (Fig. **12**) inhibits carcinoid cell growth and promotes TRAIL-induced apoptosis [110]. Since ionizing radiation has been shown to up-regulate the *TRAILR1* and *-2* genes and/or the *TRAIL* gene through p53- and p63-dependent mechanisms causing an autocrine or paracrine type of apoptosis [103], a combination of radiation and a TRAIL analogue and/or a PPAR-γanalogue might be valuable as therapy.



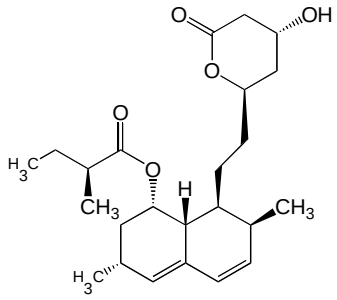
Pioglitazone

Fig. (12). Agonist of PPAR-γ.

By activating SAPK/JNK, the Fas death receptor was expressed and shown to facilitate apoptosis in irradiated leukemia cells [111]. Post-irradiation events such as membrane damage (by anti-oxidant inhibition of lipid peroxidation) further induce caspase 3-dependent apoptosis, with no change in expression of the *BCL2* and *BAX* genes [112]. The extent to which Fas receptors are expressed in carcinoids is, however, unclear.

Possible Interaction Between Radiation and Other Signalling Pathways

Over-expression of *RAS* has been shown to increase resistance to radiation [113, 114]. Radio-sensitivity may thus be manipulated in pathways upstream or downstream from RAS. One method of increasing the radiosensitivity is by inhibition of RAS activation, e.g. by using *lovastatin* and farnesyl-transferase inhibitors (Fig. **13**) to block the processing of RAS [115, 116]. *EGFR* expression and AKT phosphorylation are also associated with the response to radiation, and inhibition of EGFR, RAS, PI3K, and AKT has been shown to increase the radio-sensitivity of cancer cell lines. PI3K is a mediator of RAS-induced radiation resistance. EGFR, RAS, and PTEN can also regulate the PI3K pathway. Molecular-based radiosensitization could thus be directed against signals that are common to these pathways [113].



Lovastatin

Fig. (13). Inhibitor of RAS activation.

As mentioned above, neuroendocrine tumours frequently express VEGF, which is correlated to tumour progression [36]. VEGF leads to increased angiogenesis and higher oxygen concentration. Higher oxygen levels in the tumour tissue enhance the cytotoxic effect of radiation (oxygen effect). On the other hand, radiation therapy also increases expression of VEGF, due to radiation stress of the tumour, and may contribute to resistance to treatment through higher cell survival and proliferation. Blocking of the radiation-mediated increase in VEGF with anti-VEGF therapy could therefore increase the destruction of tumour cells and produce additive anti-tumour effects to that of radiation alone. Clinical trials designed to address this issue are in progress.

Epigenetic Therapy and Targeting of Notch Signalling

Changes in gene expression due to mechanisms other than altered DNA sequences are termed epigenetic changes. During tumorigenesis, global hypo-methylation and promoter hypermethylation are commonly observed. DNA methylation of cytosines occurs at CpG sites (4% of the genome), i.e. CG dinucleotide clusters in small sequences of DNA mostly in the gene promoter regions. The methylation is a covalent addition of a methyl group to the 5'-carbon of the cytosine ring, which results in 5'-methylcytosine. DNA hypermethylation contributes to tumour development by predisposing to mutations at methylated CpG sequences and by silencing of specific genes, repressing gene transcription by inhibiting the binding of transcription factors, or by recruiting methyl CpG-binding proteins [117]. Several genes are hyper-methylated in ileal carcinoids, e.g. *RUNX3*, O^6*-MGMT*, *RASSF1A*, *p14*, and *CTNNB1*. Some of these are suppressor genes associated with advanced disease [118-120]. The cytidine analogues 5-azacytidine and 5-azadeoxycytidine are demethylating agents currently being tested as treatment for patients with different tumour types. Global hypo-methylation influences the repetitive interspersed nuclear elements (45% of the genome), which normally protects against harmful retroviral sequences. Loss of this function may lead to loss of heterozygosity and gene rearrangements [117]. Global hypo-methylation is more common in carcinoids than in EPT, and is ssociated with features such as loss of chromosome 18, methylation of *RASSF1A*, and lymph node metastasis [121].

Another epigenetic mechanism is modification of histones, which are proteins associated with DNA in the chromatin complex. As part of gene regulation the histones are acetylated, or deacetylated, on lysine residues in the N-terminal tail. These reactions are catalyzed by enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. The removal of acetyl groups increases the positive charge of the histone tails, which promotes binding between the nucleosome and the negatively charged phosphate groups of the DNA backbone. The increased DNA binding condenses the chromatin structure and prevents transcription of genes. Down-regulation of tumour suppressor genes, evasion from apoptosis, and reduced differentiation are hallmarks of cancer that can be due to abnormal epigenetic control caused by de-regulated HDAC activity. HDAC inhibitors are emerging as anti-cancer drugs with the capacity to prevent silencing of genes and to reverse crucial steps in tumorigenesis. These inhibitors can thus induce apoptosis and differentiation, suppress proliferation and angiogenesis, and enhance the host immune system [122]. However, HDAC can also modulate the acetylation of cytoplasmic proteins, e.g. p53, the STAT family, E2F, tubulin, and heat shock protein 90 (HSP90), which are involved in a variety of cellular mechanisms. Thus, HDAC inhibitors can act through both epigenetic and non-epigenetic mechanisms. HDAC inhibitors have a broad molecular range of targets and new inhibitors are continuously under development.

Notch signalling is an important developmental pathway and plays a central role in stem cell maintenance, cell fate decisions, and differentiation. The Notch protein is a cell membrane receptor that becomes activated upon ligand binding to the extracellular domain. Induced proteolytic cleavage by γ-secretase releases the intracellular domain of the Notch receptor, which enters the cell nucleus, binds to DNA, and alters gene expression. Depending on cellular context, Notch can act either as a tumour suppressor or as an oncogene. In hepatocellular carcinoma and in neuroendocrine tumours, e.g. SCLC, medullary thyroid cancer, and gastrointestinal carcinoids, Notch signalling is very low or absent and is associated with growth suppression. In these tumour types, Notch is considered to be a tumour suppressor [123-126]. Notch signalling is a negative regulator of the expression of achaete-scute complex homolog 1 (ASCL1); the ASCL1 protein is expressed in both progenitor cells and neuroendocrine tumour cells.

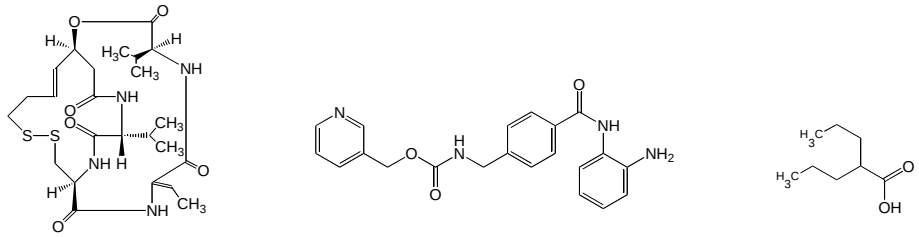
The amyloid precursor-like protein 1 (APLP1) belongs to the amyloid precursor protein (APP) family, which, like Notch, are activated by γ-secretase. There is documented cross-talk between the APP proteins and Notch. One mechanism occurs through interactions between the cleaved fragment of APP and cytoplasmic inhibitors of the Notch receptor [127]. The second way is through interaction between APP and Notch by heterodimerization [128]. Gastrointestinal neuroendocrine tumours and the GOT1 cell line have elevated expression of APLP1 in comparison with non-neuroendocrine gastrointestinal tumours. This finding raises the possibility that signals from the APP and Notch protein families may interact and regulate the differentiation of neuroendocrine cells. The increased expression of APLP1 in hepatic metastases in comparison to that in primary ileal carcinoids indicates that it may have a role in invasiveness and spread [129].

The HDAC inhibitor *valproic acid (VPA)* is a branched fatty acid with an established safety profile as an anti-epilepsy drug (Fig. **14**). Stockhausen and collaborators showed that VPA caused elevated levels of Notch1 in neuroblastoma cells [130]. Recent work on BON cells and pulmonary carcinoid cells has shown that the drug causes cell cycle arrest in G1 and suppresses the synthesis of CgA. These effects were found to be associated with activation of Notch1 (increased full-length Notch1 and its active intracellular domain). Reporter assays verified that VPA-induced Notch1 was functionally active (with binding to the centromere-binding factor and the ASCL1 promoter); siRNA against Notch1 inhibited the drug-induced effects on proliferation [131].

HDAC inhibitors alone, or in combination with other anti-cancer drugs, are currently in clinical trials for a wide range of tumour types. However, until now clinical studies on patients with ileal carcinoids using HDAC inhibitors have been very limited. A phase-I study has been conducted using *entinostat (MS-275)* in treatment of patients with advanced solid tumours including carcinoids [132] (Fig. **14**). A phase-II study using *romidepsin (depsipeptide/FK228)* in patients with locally advanced or metastatic neuroendocrine tumours was terminated due to an unexpected high number of serious cardiac adverse events [133] (Fig. **14**).

Targeting of Chemokine Receptors

Directional migration of cells during embryonic development, during tissue repair, and during the process of homing of hematopoetic stem cells to distinct niches is regulated by chemokines. Chemokines bind and activate specific G protein-coupled chemokine receptors. The chemokine receptors are divided into different families according to structure: the CXC-, CC-, CX3C-, and XC- chemokine receptors. The chemokine CXCL12 (stromal cell-derived factor 1/SDF1) activates the CXC chemokine receptor CXCR4. *In vitro*, CXCR4-expressing cells migrate towards a gradient of CXCL12. *In vivo*, CXCL12 is secreted by mesenchymal fibroblasts, osteoblasts, and endothelial cells. There is substantial evidence to suggest that CXCL12-CXCR4-mediated migration is crucial for the metastatic process and tissue-specific spread of breast and prostate cancer [134-138]. To date, three types of neuroendocrine tumours have been shown to express CXCR4: SCLC, neuroblastoma, and ileal carcinoids [139-141]. In ileal carcinoid cells (GOT1), we recently found that stimulation of CXCR4 by its ligand CXCL12 markedly activates the ERK1/2 signalling pathway and promotes cell migration under hypoxic conditions [139]; CXCR4 is thus a potential marker of tumour stem cells and hypoxic tumour cells [142]. Pre-clinical testing of a number of CXCR4-antagonistic peptides or small molecules, and antibodies to CXCR4 or CXCL12, has been conducted in mouse tumour models with promising results [138, 143-147]. Some of these small molecules are currently being investigated in acute myeloid leukaemia and multiple myeloma, but not in neuroendocrine tumours [148].



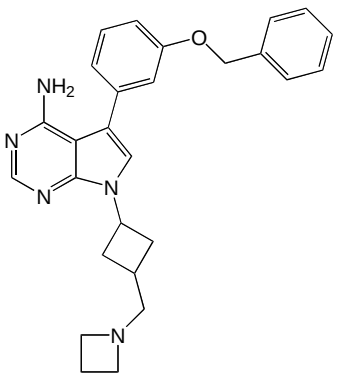
Romidepsin **Entinostat** **Valproic acid**

Fig. (14). Inhibitors of HDAC.

Targeting of IGF1R, EGFR, and TGF Signalling

Insulin-like growth factor 1 (IGF1) signalling has emerged as an important mechanism in the development and progression of cancer [149, 150]. A key regulator in this signalling pathway is IGF1R, which is widely expressed in malignant tumours including neuroendocrine tumours [151]. Over-expression of IGF1R in tumour cells causes increased proliferation and resistance to chemotherapy and apoptosis. IGF1R is a tetrameric membrane protein with an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Activation of IGF1R is achieved by phosphorylation of the tyrosine kinase, which in turn leads to activation of the AKT and MAPK signalling pathways. Targeting of IGF1 signalling has therefore emerged as a promising therapeutic strategy [149].

The role of IGF1 signalling has been investigated in the pancreatic carcinoid cell line BON; BON cells secrete both IGF1 and IGFBP2 and they express functional IGF1R [152]. Exogenously supplied IGF1 was found to stimulate BON cells to secrete CgA and to proliferate. It has been suggested that endogenous IGF1 has a role as an autocrine regulator of carcinoid tumour cell growth. The effects of IGF1 stimulation were shown to be mediated by activation of PI3 kinase, p70 ^{s6k}, and MAPK/ERK [152], and also by activation of AKT and mTOR [153]. Inhibition of IGF1R phosphorylation by *NVP-AEW541* was found to cause cell cycle arrest, activation of caspase 3, and apoptosis in BON cells [154] (Fig. **15**). Forced activation of the RAF1 signalling pathway in BON cells was also shown to interfere with MEK1/2 and IGF1 signalling, causing reduced production of CgA. However, to date IGF1 signalling has not been evaluated in the small intestinal carcinoid cell lines KRJ-I and GOT1, although an autocrine role for IGF1 has been demonstrated in primary cell cultures of ileal carcinoids [155]. At present, there are no published clinical studies on the effectiveness of IGF1R inhibition in ileal carcinoids.



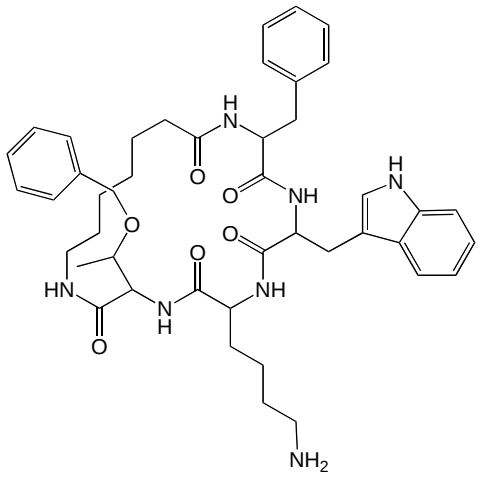
NVP-AEW541

Fig. (15). Inhibitor of IGF1R.

The epidermal growth factor receptor (EGFR) is frequently activated in malignant tumours e.g. colorectal carcinoma, NSCLC, head and neck squamous cell carcinoma, and pancreatic cancer [156]. Activated EGFR has been successfully targeted in these tumours using small molecules that inhibit RET or monoclonal antibodies that interfere with receptor dimerization. Constitutive activation of the EGFR receptor in tumours is the result of gene amplification or mutations in the extracellular part of the receptor, or in the tyrosine kinase domain. Mutations in EGFR strongly influence the responsiveness to TKI, while mutations in downstream effector molecules, e.g. *KRAS*, *BRAF*, *PIK3AC*, or *PTEN* confer resistance to EGFR-targeted therapy. The EGFR signalling pathway has been investigated in pancreatic and ileal carcinoid cells (BON and KRJ-I cells, respectively). EGFRs were demonstrated in BON cells, and exogenous administration of EGF or TGFα induced a proliferative response [53, 157, 158]. Treatment with the EGFR inhibitors *gefitinib* or *AG1478* induced an anti-proliferative response due to cell cycle arrest and apoptosis. In KRJ-1 cells, however, TGFα induced cell proliferation whereas EGF did not [53]. Treatment with gefitinib, on the other hand, inhibited proliferation of KRJ-I cells [159]. Furthermore, studies on primary cell cultures from ileal carcinoids have found an autocrine stimulatory effect of TGFα that could be blocked by neutralizing EGFR antibodies [160]. The experimental data thus clearly indicate that EGFR signalling is of major importance in carcinoid tumour cells. Clinical studies on patients with carcinoid tumours have demonstrated disease stabilization, or even regression of tumours, after EGFR inhibition with gefitinib as monotherapy [18].

The transforming growth factor β (TGFβ) family has 33 members; these control developmental processes as well as adult homeostasis. TGFβs regulate tissue homeostasis by their ability to induce cell cycle arrest and apoptosis, and to preserve genomic stability. In malignant tumours, the TGFβ signalling pathway is frequently perturbed, causing resistance to the cytostatic activities of TGFβ. The oncogenic properties of TGFβ are poorly understood, but include regulation of the tumour microenvironment, of angiogenesis, of the epithelial-mesenchymal transition, and of metastasis formation [161]. TGFβ ligands signal via receptor serine/threonine kinases (TGFβR) that undergo phosphorylation upon ligand binding and activate intracellular SMAD effectors. Oligomeric SMAD complexes translocate to the nucleus, associate with chromatin, and regulate gene transcription [162]. The TGFβ signalling pathway has been investigated in pancreatic (BON) and ileal (KRJ-I) carcinoid cells. Surprisingly, BON cells and KRJ-I cells showed opposite proliferative responses to exogenous TGFβ. BON cell growth was inhibited, while KRJ-I cell growth was stimulated after administration of TGFβ [53, 163]. Stimulation of BON cells with TGFβ induced G1 cell cycle arrest, reduced expression of *MYC*, and increased expression of $p21^{WAF1/CIP1}$. Furthermore, TGFβ was found to be secreted from tumour cells, suggesting that there is an autocrine growth inhibition by TGFβ [164]. The relationship be-

tween TGFβ and somatostatin signalling has been investigated in BON cells [165]. Treatment with TGFβ caused increased expression of somatostatin and SSTR2 in tumour cells. Administration of the SSTR antagonist *cyclo-somatostatin* abolished the growth inhibition induced by TGFβ**,** indicating that the growth-inhibitory effect of TGFβ is mediated by SSTRs (Fig. **16**). The TGFβ signalling pathway has also been characterized in KRJ-I cells. In these cells, TGFβ stimulation did not induce nuclear translocation of SMAD or growth inhibition; on the contrary, TGFβ induced cell proliferation [166]. TGFβ-stimulated growth was associated with SMAD2 phosphorylation, but with reduced SMAD4 expression and increased SMAD7 expression. The TGFβ target gene $p21^{WAF1/CIP1}$ was down-regulated, while *c-MYC* was up-regulated. These molecular changes together with phosphorylation of ERK1/2 partly explain the proliferative response of KRJ-I cells upon TGFβ stimulation. Identification of an aberrant TGFβ signalling pathway in ileal carcinoids may provide novel targets for therapy. Anti-TGFβ compounds are under development and have proven efficacy in pre-clinical studies [167]. However, there is still a lack of clinical studies on carcinoid tumours.



Cyclo-somatostatin

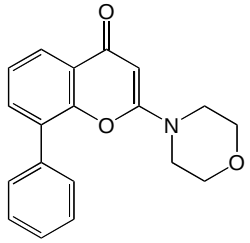
Fig. (16). SSTR antagonist.

A number of additional growth factors/receptors have been identified and functionally characterized in carcinoid cells, including nerve growth factor receptor (NGFR) and connective tissue growth factor (CTGF) [53, 168]. The exact role of these factors and receptors in carcinoid growth regulation and metastasis remains to be clarified, as does their suitability as targets for anti-cancer therapy.

Targeting of PI3K-AKT Signalling

In several tumour types, including the neuroendocrine tumours such as medullary thyroid carcinoma, up-regulation of the PI3K-AKT signalling pathway can promote tumour growth and inhibit apoptosis [169]. AKT (protein kinase B) is the key effector; it must be phosphorylated at a threonine and serine site for full activity. This is carried out by PI3K and phosphate-dependent dehydrogenase-1. Phosphorylated AKT in turn controls several caspases and transcription factors of the Forkhead family. AKT occurs in three isoforms; of these, AKT1 is the main one, AKT2 occurs in insulin-responsive tissues, and AKT3 occurs in the central nervous system [170].

Studies on human pulmonary carcinoid cells (NCI-H727) subjected to blockade with *LY294002*, a well-characterized PI3K inhibitor, or subjected to silencing with AKT1 siRNA showed reduced growth and reduced expression of neuroendocrine markers (CgA and ASCL-1) but to different degrees (85% as opposed to 31%) [171] (Fig. **17**). The study did not address whether anti-proliferative or apoptotic mechanisms were activated. Previous studies on endocrine-related tumours have shown that AKT1 regulates migration and invasiveness in breast cancer [172] and neuroendocrine differentiation in prostate cancer [173]. Of special interest for therapy with ^{177}Lu-octreotate is that targeting of AKT1 enhances the radiation toxicity of human tumour cells by inhibiting the DNA-PK-dependent DNA double-strand break repair [174]. PI3K-AKT signalling has been implicated also in solid tumours and haematological malignancies, and clinical trials with PI3K-AKT inhibitors have been initiated. No experimental studies on gastrointestinal carcinoids have been reported.



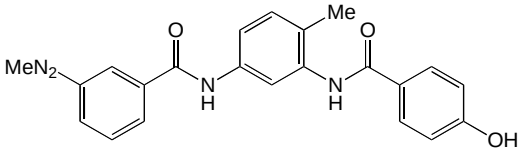
LY294002

Fig. (17). Inhibitor of PI3K-AKT.

Targeting of RAS/RAF1 Signalling

In this pathway, RAS activates RAF1, which is a serine/threonine kinase, resulting in phosphorylation of MEK 1/2 and ERK 1/2. The pathway can be constitutively activated by oncogenic RAS/RAF isoforms, or over-expression of *RAS/RAF* [175]. This pathway is commonly activated in gastrointestinal carcinomas, but mutations in *RAS* are unusual in intestinal carcinoids [176]. *BRAF* mutations are also rare in neuroendocrine tumours, but the activating protein RAP1 is frequently expressed [177]. In a recent report, RAF1/BRAF signalling was shown to be activated in neuroendocrine tumours, which makes this pathway a putative therapeutic target [178]. Notably, RAF1 activation in medullary thyroid cancer leads to reduced tumour growth and reduced levels of neuroendocrine tumour markers [125].

Pharmacological activation of RAF1 by *ZM336372* was found to induce progressive phosphorylation of RAF1, MEK 1/2, and ERK 1/2 in both human pulmonary carcinoid cells and BON (pancreatic carcinoid) cells, accompanied by reduced levels of CgA and the transcription factor ASCL1 [179] (Fig. **18**). The treatment suppressed proliferation, possibly through induction of cell cycle inhibitors such as p18 and p21. The results must be interpreted with some caution, since the drug may phosphorylate proteins other than RAF 1 [180]. The basic helix-loop transcription factor ASCL1 was previously shown to be highly expressed in neuroendocrine tumour cells [181]. MASH-1 is the mouse homologue of ASCL1 and is important for foetal development of certain neurons, thyroid C-cells, and adrenal chromaffin cells [182-184]. Several of the studies cited indicate that ASCL1 controls the expression of neuroendocrine granule proteins, i.e. depletion of ASCL1 is associated with reduction of CgA and synaptophysin expression. Pharmacological RAF1 activation may therefore be a future therapeutic option with the potential to alleviate hormonal symptoms. No experimental studies have been performed on midgut carcinoid tumour cells.



ZM336372

Fig. (18). Activator of RAF1.

CONCLUSION

Targeting of growth factors and their receptors, angiogenic factors, and hormone receptors expressed on neuroendocrine tumour cells provides new therapeutic options. The signal transduction in carcinoid tumour cells is not fully understood, but experimental interference with specific pathways, e.g. mTOR, PI3K, RAS/RAF, and Notch, offers some promise aside from more general mechanisms such as inhibition of the proteasome and HDAC. The combination of several therapeutic strategies seems rational, since it gives tumour cells limited options for escape. In clinical studies, combination therapies (based on TKIs) have had acceptable toxicity. Radionuclide therapy via highly expressed SSTRs has been shown to be effective clinically in limited, but disseminated tumour disease. New knowledge gained concerning the apoptotic pathway, PARP inhibition, and death receptors could lead to potentiated radiotherapeutic effects.

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ABBREVIATIONS

ADP = Adenosine diphosphate

AKT = v-Akt murine thymoma viral oncogene homolog, a serine-threonine protein kinase, also known as protein kinase B

AKT1 = Involved in cellular survival pathways, a major factor in many cancers

AKT2 = Important signalling molecule in the insulin signalling pathway

AKT3 = Occurs predominantly in the central nervous system

AG1478 = An EGFR inhibitor

AMG706 = A pan-VEGFR inhibitor, a nicotinamide

APLP1 = Amyloid precursor-like protein 1

APP = Amyloid precursor protein

ASCL1 = Achaete-scute complex homolog 1

ATP = Adenosine triphosphate

BAX = BCL2-associated X protein

BCL2 = A family of genes and proteins that are either pro- or anti-apoptotic

bFGF = Basic fibroblast growth factor

BON = A human pancreatic carcinoid cell line

BRAF = V-raf murine sarcoma viral oncogene homolog B1

cAMP = Cyclic adenosine monophosphate, a second messenger

caspase = Cysteine-aspartic acid protease, involved in, e.g., apoptosis, and necrosis

CDKN1A = Cyclin-dependent kinase inhibitor 1A, also known as p21, WAF1, or CIP1; a cell cycle inhibitor

cFLIP = FLICE (FADD-like IL-1β-converting enzyme) inhibitory protein, a protease-deficient caspase homologue

CgA = Chromogranin A

CHS828 = A pyridyl cyanoguanidine with anti-tumour activity

CIP1 = Cyclin-dependent kinase inhibitor 1A, also known as p21, WAF1, or CDKN1A; a cell cycle inhibitor

c-MYC = A gene encoding for a transcription factor regulating expression of many genes

CNDT2 = A human ileal (midgut) carcinoid cell line

CNS = Central nervous system

COX-2 = Cyclooxygenase-2

CpG = Cytosine-phosphate-guanine dinucleotide sequnce of DNA

c-RAF = A gene that codes for the protein kinase c-RAF; also called RAF1

CTGF = Connective tissue growth factor

CTNNB1 = A gene coding for β-catenin

CX3C = A type of chemokine

XC = A family of chemokines

CC = A family of chemokines

CXC = A family of chemokines

CXCR4 = Chemokine (C-X-C motif) receptor 4, also known as fusin

DNA = Deoxyribonucleic acid

DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, a chelate

E2F = A group of genes that codes for a family of transcription factors, involved in cell cycle regulation and DNA synthesis

EGF = Epithelial growth factor

EGFR = Epithelial growth factor receptor

EPT = Endocrine pancreatic tumour

EORTC = European Organisation for Research and Treatment of Cancer

ERK = Extracellular signal-regulated kinase; also known as mitogen-activated protein kinase, MAPK

ERK1 = Also known as MAPK3, an enzyme encoded by the *MAPK3* gene

ERK2 = Also known as MAPK1, and p42MAPK, an enzyme encoded by the *MAPK1* gene

FADD = Fas-associated protein with death domain

FasR = Fas receptor, one of the death receptors

FGFR1 = Fibroblast growth factor receptor 1, also known as basic fibroblast growth factor receptor 1, fms-related tyrosine kinase-2/Pfeiffer syndrome, and CD331

FK228 = An HDAC inhibitor, also called depsipeptide

FK866 = An NAMPT inhibitor

FLT3 = Fms-like tyrosine kinase 3

FOLFOX = A chemotherapy regimen consisting of 5-fluorouracil, leucovorin, and oxaliplatin

G1 = Gap 1, one of the cell cycle phases

GIST = Gastrointestinal stromal tumour

GMX1778 = An inhibitor of nicotinamide phosphoribosyltransferase (NAMPT)

GOT1 = A human ileal (midgut) carcinoid cell line

GOT2 = A human medullary thyroid carcinoma cell line

Gy = Gray, unit of absorbed dose

HIF-1 = Hypoxia-inducible factor 1
HSP90 = Heat shock protein 90
HAT = Histone Acetyltransferase
HDAC = Histone Deacetylase
IC_{50} = Half maximal inhibitory concentration
IGF = Insulin-like growth factor
IGF1 = Insulin-like growth factor 1
IGF1R = Insulin-like growth factor 1 receptor
IGFBP2 = Insulin-like growth factor binding protein 2
IGFBP3 = Insulin-like growth factor binding protein 3
IgG = Immunoglobulin G
IκB = Inhibitory protein of NF-κB
IKK = Inhibitory protein of NF-κB kinase
In = Indium
JNK = C-Jun N-terminal kinase
KIT = A tyrosine kinase receptor, also known as C-kit or CD117
KRJ-1 = A human ileal (midgut) carcinoid cell line
KRAS = V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAR = Long acting release
Lu = Lutetium
LY294002 = A selective phosphatidylinositol 3-kinase (PI3K) inhibitor
MASH-1 = The mouse homologue of ASCL1
MAP = Mitogen-activated protein
MAPK = Mitogen-activated protein kinase
MEK1 = Dual-specificity mitogen-activated protein kinase kinase 1, also known as ERK activator kinase 1 or MAPK/ERK kinase 1
MEK2 = Dual-specificity mitogen-activated protein kinase kinase 2, also known as ERK activator kinase 2, or MAPK/ERK kinase 2
MGMT = Methylguanine-DNA methyltransferase
mRNA = Messenger ribonucleic acid
MS275 = Entinostat, an HDAC inhibitor
mTOR = Mammalian target of rapamycin
MYC = V-myc myelomatosis viral oncogene homolog, a transcription factor regulating cell cycle progression and apoptosis
NAD^+ = Nicotinamide adenine dinucleotide
$NADP^+$ = Nicotinamide adenine dinucleotide phosphate
NADPH = The reduced form of NADP+
NAMPT = Nicotinamide phosphoribosyltransferase
NCI = National Cancer Institute
NCI-H727 = A human pulmonary carcinoid cell line
NF-κB = Nuclear factor-κB
NGFR = Nerve growth factor receptor
NIH = National Institutes of Health
NM = Nicotinamide
NMNAT = Nicotinamide mononucleotide adenyltransferase
NMN = Nicotinamide mononucleotide
NMNAT = Nicotinamide mononucleotide adenyltransferase
NO = Nitric oxide
NSCLC = Non-small cell lung carcinoma
NVP-AEW541 = A selective IGF1R inhibitor
NYH = A human small cell lung carcinoid cell line
O^6-MGMT = O^6-methylguanine-DNA methyltransferase, interacts with estrogen receptor alpha
p14 = The fusion-associated small transmembrane (FAST) protein
p18 = Cyclin-dependent kinase 4 inhibitor C, a cell cycle inhibitor
p21 = Cyclin-dependent kinase inhibitor 1A, also known as WAF1, CIP1, or CDKN1A; a cell cycle inhibitor
p38 = A class of mitogen-activated protein kinases involved in cell differentiation and apoptosis
p50 = A mature subunit of NF-κB
p53 = Protein 53, or tumour protein 53, is a tumour suppressor protein; also known as cellular tumour antigen p53, antigen NY-CO-13, phosphoprotein p53, transformation-related protein 53 (TRP53), or tumour suppressor p53
p63 = Tumour protein p63, also known as transformation-related protein 63, a member of the p53 family of transcription factors
$p70^{s6k}$ = p70 ribosomal S6 protein kinase
PARP = Poly (ADP-ribose) polymerase; involved in, e.g., DNA repair and apoptosis
PARP1 = Poly (ADP-ribose) polymerase 1
PARP2 = Poly (ADP-ribose) polymerase 2
PDGF = Platelet-derived growth factor
PDGFA = PDGF isoform A
PDGFB = PDGF isoform B
PDGFR = Platelet-derived growth factor receptor, a receptor tyrosine kinase
PDGFRA = PDGFR alpha isoform
PI3K = Phosphatidylinositol 3 kinase, involved in the PI3K/AKT signalling pathway
PIK3CA = The catalytic subunit of PI3K
PlGF = Placental growth factor
PlGF1 = Placental growth factor 1
PlGF2 = Placental growth factor 2
PPAR-γ = Peroxisome proliferator-activated receptor gamma
PTEN = Phosphatase and tensin; acts as a tumour suppressor gene
RAF1 = A serine/threonine kinase, also known as RAF or c-RAF
RAP1 = Ras-proximate-1, a small GTPase involved in signal transduction
RAS = A family of genes encoding small GTPases; involved in cell signal transduction
RASSF1A = Ras association domain-containing protein 1, encoded by the RASSF1 gene
RB = Retinoblastoma protein, also known as pRb; a tumour suppressor protein encoded by the RB1 gene

RECIST	=	Response evaluation criteria in solid tumours, adopted by, e.g., EORTC, NCI of the USA, and NCI of Canada
RelA	=	The *RELA* gene codes for the transcription factor p65, part of the NF-κB complex
RET	=	Receptor tyrosine kinase, a proto-oncogene
ROS	=	Reactive oxygen species
RUNX3	=	Runt-related transcription factor 3
SCLC	=	Small cell lung carcinoma
SAPK	=	Stress-activated protein kinase, also known as MAPK9
siRNA	=	Small interfering RNA, also called short interfering RNA or silencing, RNA
SMAD	=	A class of proteins that modulate the TGFβ signalling
SMAD2	=	One of the receptor-regulated SMADs
SMAD4	=	The common-mediator SMAD
SMAD7	=	One of the inhibitory SMADs
SOM230	=	A pan-somatostatin receptor analogue
SSTRs	=	Somatostatin receptors, consisting of 5 subtypes, SSTR1-SSTR5
STAT	=	Signal transducers and activator of transcription protein; also called signal, transduction and transcription protein
STS	=	A human ileal (midgut) carcinoid cell line
TGFα	=	Transforming growth factor α
TGFβ	=	Transforming growth factor β
TGFβR	=	Transforming growth factor β receptor; a serine/threonine kinase receptor
TKI	=	Tyrosine kinase inhibitor
TNF	=	Tumour necrosis factor
TRAIL	=	TNF-related apoptosis-inducing ligand, also designated CD253; the natural ligand of TRAIL receptors
TRAILR	=	TRAIL receptors
TRAILR1	=	TRAIL receptor 1, also called DR4; one of the death receptors
TRAILR2	=	TRAIL receptor 2, also called DR5; one of the death receptors
TT	=	A human medullary thyroid carcinoma cell line
Tyr	=	Tyrosine
UPP	=	Ubiquitin-proteasome pathway
VEGF	=	Vascular endothelial growth factor; exists in 5 subtypes: VEGFA-VEGFE
VEGFR	=	Vascular endothelial growth factor receptor
VEGFR1	=	Vascular endothelial growth factor receptor 1, also called FLT1
VEGFR2	=	Vascular endothelial growth factor receptor 2, also called KDR/FLK1
VEGFR3	=	Vascular endothelial growth factor receptor 3, also called FLT4
VPA	=	Valproic acid
WAF1	=	Cyclin-dependent kinase inhibitor 1A, also known as p21, CIP1 or CDKN1A, a cell cycle inhibitor
ZM336372	=	A RAF1 activator

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