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Two novel combination therapies of glioblastoma multiforme from a Cx43 perspective

Studies of *in vitro* models

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Det är i motvind som man kan lyfta och flyga

Elfar Ulfarsson f 1965

ABSTRACT

The group of patients suffering from the malignant brain tumour glioblastoma multiforme (GBM) have a median survival of only about one year. Thus, new treatment strategies are urgently needed.

Gap junctions are intercellular channels, permitting intercellular transfer of water soluble substances. They are vital for cellular homesostasis, proliferation and differentiation. Gap junctions are composed of multimeric proteins termed connexins (Cxs), of which Cx43 is the most ubiquitous. Cx43 serves as a tumour suppressor independent of its ability to form gap junctions.

Herpes simplex virus thymidine kinase (HSV-tk) mediated suicide gene therapy has shown promising results in preclinical research, but has as yet failed to prove clinical efficacy. Intriguingly, neural progenitor cells (NPCs) have shown tropism for intracranial tumours and could be exploited for improving the delivery of therapeutic genes or gene products.

Epigenetic regulators, such as histone deacetylase inhibitors (HDACi) and receptor tyrosine kinase inhibitors (RTKIs), including gefitinib and vandetanib, have recently entered clinical trials.

In the present study, primary cultures of GBM cells were established and showed positivity for neuroepithelial markers. Additionally, they were Cx43 positive and functional gap junctions were demonstrated.

The bystander effect in the HSV-tk/ganciclovir (HSV-tk/GCV) suicide gene therapy system was attenuated by a specific gap junction inhibitor, AGA, showing that this phenomenon is dependent on gap junction coupling in glioma cells.

The HDAC inhibitor 4-phenylbutyrate (4-PB) up regulated Cx43 and enhanced gap junction communication (GJC), a strategy which could have potential for improving efficacy of suicide gene therapy. NPCs exposed to 4-PB also increased Cx43 expression and gap junction formation. This, together with the propensity of NPCs of tracking down tumour cells, could add a new dimension to gene therapy.

Cooperative antitumoural effects were evident in combination treatment of 4-PB and the RTKIs gefitinib and vandetanib. These RTKIs showed an up regulating effect on the tumour suppressor Cx43, which could show to be of importance in the downstream effects of RTKIs.

Keywords: glioma, gap junction, Cx43, bystander effect, HSV-tk, HDAC inhibitor, tyrosine kinase inhibitor

LIST OF PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV):

I. Gap junction-mediated bystander effect in primary cultures of human malignant gliomas with recombinant expression of the HSVtk gene.
Exp Cell Res. 2003 Apr 1;284(2):185-95.
Asklund T, Appelskog IB, Ammerpohl O, Langmoen IA, Dilber MS, Aints A, Ekström TJ, Almqvist PM.

II. Histone deacetylase inhibitor 4-phenylbutyrate modulates glial fibrillary acidic protein and connexin 43 expression, and enhances gap-junction communication, in human glioblastoma cells.
Eur J Cancer. 2004 May;40(7):1073-81.
Asklund T, Appelskog IB, Ammerpohl O, Ekström TJ, Almqvist PM.

III. HDAC inhibition amplifies gap junction communication in neural progenitors: potential for cell-mediated enzyme prodrug therapy.
Exp Cell Res. 2007 Aug 1;313(13):2958-67. Epub 2007 May 22.
Khan Z, Akhtar M, **Asklund T**, Juliusson B, Almqvist PM, Ekström TJ.

IV. Cooperative cytotoxicity by HDAC inhibitors and RTKIs: induction of Cx43 in GBM cells.
Asklund T, Marino A, Juhlin C, Sofiadis A, Khan Z, Larsson C, Henriksson R, Ekström TJ.
Submitted.

CONTENTS

ABBREVIATIONS

AGA	18 α glycyrrhetinic acid
Cdk	Cyclin-dependent kinase
CpG island	Cytosine-phosphate-guanine island
CT-Cx43	Carboxy terminal of Cx43
Cx43	Connexin 43
Cy3	Cyanine 3
DMSO	Dimethylsulfoxide
ECL	Enhanced chemiluminescense
EGF(R)	Epidermal growth factor (receptor)
EGFRvIII	EGF receptor variant III
ERK	Extracellular regulated kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma multiforme
GCV	Ganciclovir
GFAP	Glial fibrillary acidic protein
GJC	Gap junction communication
HAT	Histone acetyl transferase
HER	Human epidermal growth factor receptor
HDAC	Histone deacetylase
HSV-tk	Herpes simplex virus thymidine kinase
ICC	Immunocytochemistry
IF	Intermediate filament
INK4	Inhibitor of cyclin-dependent kinase 4
MAPK	MAP kinase
MAPKK (MEK)	MAP kinase kinase
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NPC	Neural progenitor cell
$p21^{WAF/CIP1}$	p21 wild type p53 associated factor/Cdk interacting protein 1
4-PB	4-Phenylbutyrate
PBS	Phosphate buffered saline
PDGF(R)	Platelet derived growth factor (receptor)
PF	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
Rb protein	Retinoblastoma protein
ROS	Reactive oxygen species
RTK(I)	Receptor tyrosine kinase (inhibitor)
SAHA	Suberoylanilide hydroxamic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TSA	Trichostatin A
VEGF(R)	Vascular endothelial growth factor (receptor)
ZO-1	Zonula occludens-1

1. BACKGROUND

1.1 Introduction

The two main cell types of the mammalian brain are neurons and glial cells.
Glia was previously considered as the scaffold (the so-called “glue of Virchow”) for the neuronal architecture in the central nervous system (CNS). During the last decades a tremendous challenge to the neuronal doctrine, in which the neuron is regarded as the basic information processing unit in the CNS, has evolved. The known functions of glia have increased exponentially and include shaping of the micro-architecture of the brain parenchyma, transmitting synaptic inputs, collaboration in signalling pathways and acting as a pool for pluripotent neural precursors and probably also for neural stem cells. While playing such a paramount role for the physiological and structural functions of the brain, glia also contributes to a wide spectrum of brain pathologies, of which the glial malignant brain tumour glioblastoma multiforme (GBM) is the most frequent and deleterious [1, 2].

The first reported extirpation of a glial brain tumour was reported by Bennett and Godlee on the 25th of November, 1884. The patient died on the 28th postoperative day of meningitis and secondary complications. No macroscopic residual tumour could be seen in the post mortem examination [3]. The initial postoperative one month mortality for gliomas was 17% in Harvey Cushing´s series and up to 40% in the hands of other surgeons [4]. It could be argued that in the dawn of neurosurgery, the disease was not necessarily more harmful to the patient than the neurosurgeon. Today, the mortality is derived almost entirely from the disease itself, as the operative mortality in modern neurosurgery is in the range of a few percent [4].

GBM is still one of the most fatal human tumours with a median survival after diagnosis of only about a year in spite of surgery, radiotherapy and chemotherapy [5, 6]. Some of the

proposed explanations for this therapeutic failure are lack of permeability of the blood brain barrier, complexity of signalling pathways, invasiveness and heterogeneity of tumour cells [7]. This necessitates development of new therapeutic strategies.

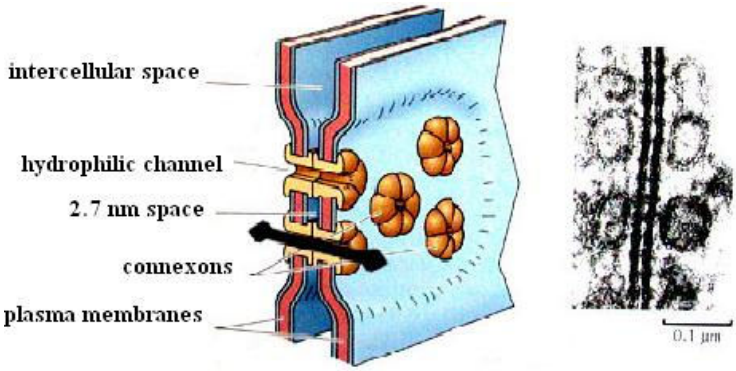
1.2 Gap junction communication

A coordinated response to external stimuli is a hallmark in the multicellular organism and vital for its adaptation to the environment. Gap junctions, key players in this context, are crucial for cellular homeostasis, proliferation and differentiation. A wide spectrum of physiological and pathological processes, such as embryogenesis, metabolic cooperation intercellular electrical coupling and carcinogenesis, are gap junction dependent [8-13].

Gap junction mediated cell-cell communication was first described by Loewenstein and co-workers in the 1960s [14, 15]. They later showed that an active temperature-sensitive src oncogene induced attenuation of gap junction coupling and thereby loss of growth control, proposing the role for gap junctions in the control of cell growth [16, 17]. Subsequently, they concluded that gap junction mediated growth control was dependent on intercellular transfer of growth regulators of low molecular weight [18].

The gap junction complex consists of two hemi channels in the contact zones of two apposing cells participating in the intercellular contact. Each hemi channel consists of six protein subunits termed connexins (Cxs), which are designated with numerical suffixes corresponding to their molecular weight in kDa. There are approximately 20 connexin members in humans. The most common and widespread connexin in mammals is connexin 43 (Cx43), with an abundant expression in the brain, heart and skin [12, 19-21]. When two cells are interconnected, the formation of gap junctions allow small water soluble substances such as ions, second messengers and metabolites with a maximum molecular weight of 1 kDa to be exchanged between cells, so called gap junction coupling or communication (GJC) (fig. 1).

Figure 1: The gap junction complex.



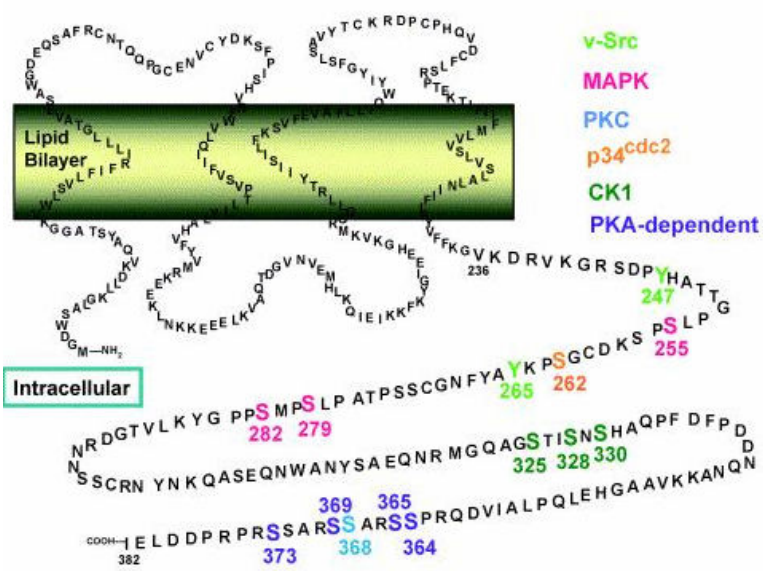
Left: *Gap junction complexes involving two juxtaposed cell membranes and their respective connexons (hemi channels) docking to each other. Arrow indicates flow of water soluble compounds between cells.*

Right: *Electron micrograph of a number of gap junction complexes.*

Furthermore, Cx43 serves as a tumour suppressor independent of its ability to form gap junctions [22-24], and is down regulated in malignant gliomas [10] and several other tumours, (reviewed in [25]). GJC is regulated by quantitative alterations of gap junctions, which is influenced by variations in expression, degradation and trafficking of Cxs, or opening or closure of existing gap junctions [26-29].

Phosphorylation of mainly serine, but also tyrosine residues, in the carboxy terminal (CT) is one of the key mechanisms for the inhibitory regulation of gap junctions (fig. 2).

Figure 2: **Cx43 and its carboxy terminal phosphorylation sites**



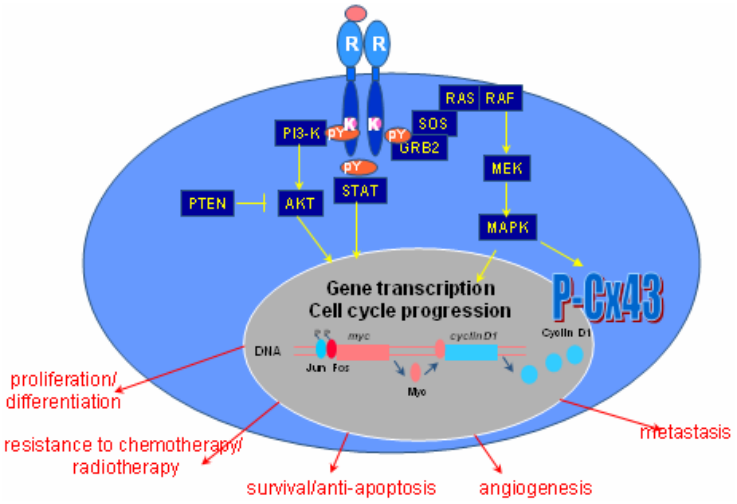
The image depicts Cx43 with its extracellular domains, hydrophobic membrane-spanning domains and cytoplasmic domains. The latter includes the amino (N)-terminal region and the carboxy terminal (CT-Cx43). Phosphorylation sites on the CT-Cx43 and their corresponding kinases are indicated with different colours [29].

Cx43 phosphorylation impacts gap junction assembly and promotes the internalization and degradation of Cx43 [27-30]. Cx43 phosphorylation increases almost five times from G0/G1 phase to M [31], while a parallel redistribution of the Cx43 protein from the plasma membrane to the cytoplasm occurs [27]. These quantitative changes of Cx43 coexist and cooperate with gap junctional gating, in which phosphorylation of the mentioned amino acid residues disrupts channel permeability. The exact molecular pathway behind gap junction

closing is still elusive, but a probable mechanism is a conformation change of the CT itself or interaction with proteins containing SH2 or PTB domains [32].

There are numerous intra- and extracellular factors and agents capable of regulating gap junction permeability, such as oncogenes, biomolecules in the signalling cascades, mitogens, hormones, cell stress etc. This regulation is mainly inhibitory in nature. Growth factors, including EGF and PDGF, exert some of their effects via gap junction inhibition by phosphorylation of the CT of Cx43 (CT-Cx43) via a mitogen activated protein kinase (MAPK) dependent pathway [30] (fig. 3).

Figure 3: The EGFR signalling pathway

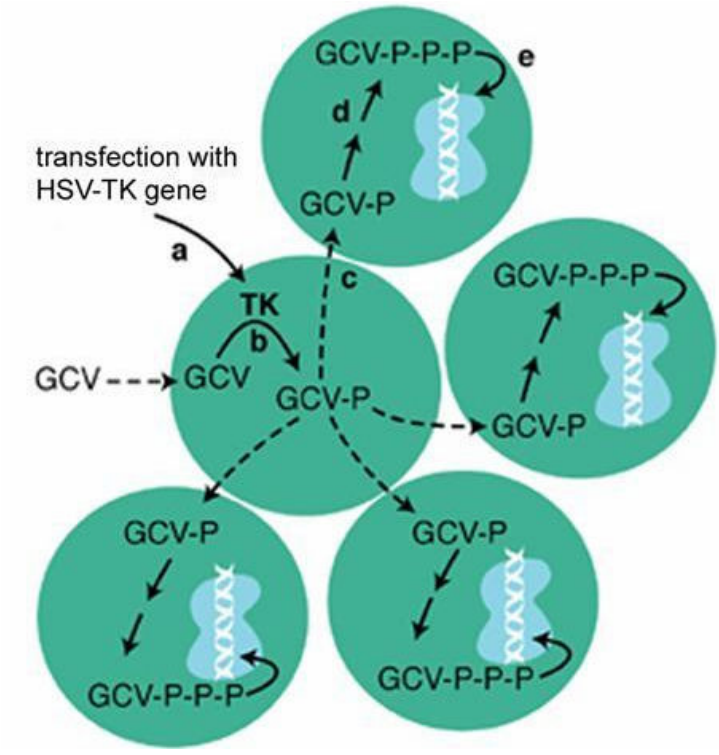


Schematic illustration of signalling initiated by ligand activation of the EGF receptor. The MAP kinase pathway is important for the phosphorylation of Cx43.

1.3 Prodrug suicide gene therapy

Gene therapy is a therapeutic strategy utilizing nucleic acids to express or knock out a gene of choice in a target cell. When the herpes simplex virus thymidine kinase (HSV-tk) gene is expressed episomally, or after its incorporation into the genome of the target cell, the added nucleoside analogue ganciclovir (GCV), is phosphorylated, first to monophosphate by the HSV-tk enzyme and subsequently to the di- and triphosphate forms, which is catalyzed by cellular kinases. The triphosphate form then incorporates into the DNA of the target cell during S-phase. This leads to strand breaks with DNA replication arrest and cell death. This paradigm is referred to as HSV-tk mediated suicide gene therapy [33, 34] (fig. 4).

Figure 4: Suicide gene therapy utilizing the HSV-tk/GCV system



(a) Delivery of the HSV-tk gene to the target cell by transfection. (b) Expression of HSV-tk phosphorylates GCV to its monophosphate form, GCV-P. (c) GCV-P diffuses to adjacent cells through gap junctions to neighbouring cell, the so-called bystander effect, where (d) additional phosphorylation occurs. (e) Oligo-phosphorylated GCV incorporates into the cellular genome leading to DNA replication arrest and cell death.

Since the endogenous thymidine kinase affinity for GCV is negligible in mammalian cells, only cells with recombinant expression of HSV-tk are able to mono-phosphorylate GCV. However, not only cells expressing HSV-tk will die, but also many of the surrounding cells, due to the so called bystander effect. This phenomenon is caused by passive diffusion of toxic (primarily mono-phosphorylated) GCV-metabolites from a cell expressing HSV-tk to its neighbours, which could include a relatively large cluster of surrounding cells. Gap junctions play a crucial role in this mechanism [35-42] and (I).

Enzyme/prodrug suicide genes, such as HSV-tk, have been evaluated with success *in vitro* and *in vivo* [35, 36, 43, 44]. The first phase I/II clinical trial using retroviral (RV)-mediated HSV-tk in malignant glioma was undertaken a decade ago [45]. Since then, a number of small clinical trials have utilized the RV suicide gene concept with few side effects, but with disappointing clinical efficacy [46-50]. This was confirmed by a large randomized phase III trial, which failed to demonstrate any prolongation of progression-free, median, or 12-month survival with HSV-tk suicide gene therapy [51].

The main hurdle in the field of clinical gene therapy is the delivery of the therapeutic gene or its products to a critical amount of tumour cells to achieve regression of tumour burden. This discouraging observation has paved the way for a promising concept in which neural stem cells/progenitor cells are utilized as vectors for gene delivery, which is discussed in the following section.

1.4 Neural progenitor cells

Stem cells, which retain the ability to proliferate throughout postnatal life, give rise to progenitor cells, which can differentiate into specialized cells. Interestingly, neural progenitor cells (NPCs) show extensive tropism for intracranial lesions including gliomas [52, 53], where they establish contacts with tumour cells. This property of tracking down target cells is appealing, as NPCs have a potential as vehicles for therapeutic genes/gene products including suicide genes [52, 54]. A possible explanation for the migratory characteristics of proliferating NPCs (in contrast to the differentiated population), is the maintenance of Cx43 expression and gap junction communication, as elegantly demonstrated in a conditional Cx43 knockout mouse model [55].

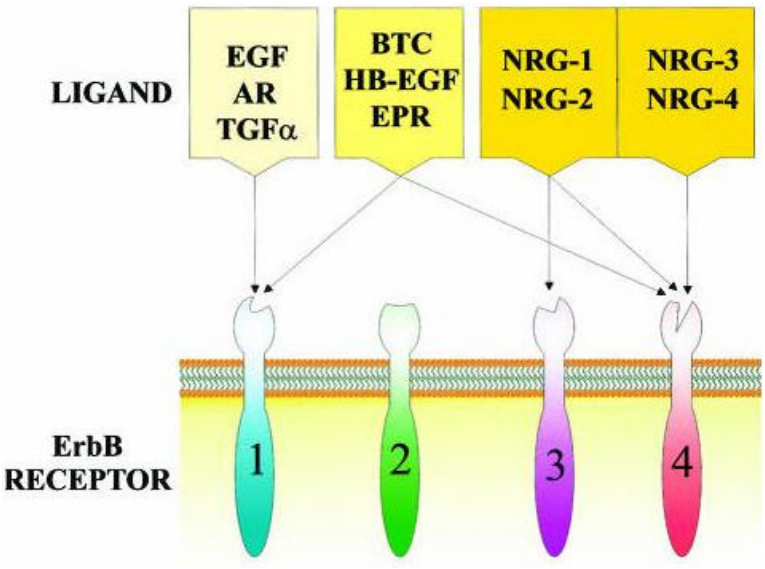
Interestingly, neural stem cells when implanted at a distant injection site from the tumour, even in the contralateral hemisphere, conferred a marked reduction of tumour size and prolonged survival in a rat model, by delivery of HSV-tk/GCV suicide gene products [56, 57].

1.5 Receptor tyrosine kinases

Receptor mediated signalling is of vital importance for the integration of external stimuli by the target cell, in order to initiate appropriate responses. Receptors of the tyrosine kinase (RTK) superfamily play a pivotal role in this context, by binding to specific growth factors and hormones, thereby generating cascades of intracellular signals, which contribute to cellular growth, proliferation and survival. In the following, two RTK receptor families are discussed: The human epidermal growth factor receptor (HER) family consisting of EGFR, HER2/neu, HER3 and HER4, and the VEGFR family including VEGFR1, VEGFR2 and VEGFR3. Ligand binding to both the HER family and the VEGFR family induces dimerization/oligomerization of the receptor, leading to autophosphorylation of tyrosine residues in its intracellular kinase domain [58-60].

The corresponding ligands to the HER family are EGF, amphiregulin (AR), transforming growth factor-α (TGF-α)- which bind specifically to EGFR- and betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR)- which exhibit dual specificity by binding to both EGFR and HER4-and the neuregulins (NRGs), where NRG-1 and NRG-2 have promiscuous affinity for HER3 and HER4, while NRG-3 and NRG-4 are specific to HER4 [58] (fig. 5).

Figure 5: Ligand specificity to the HER family

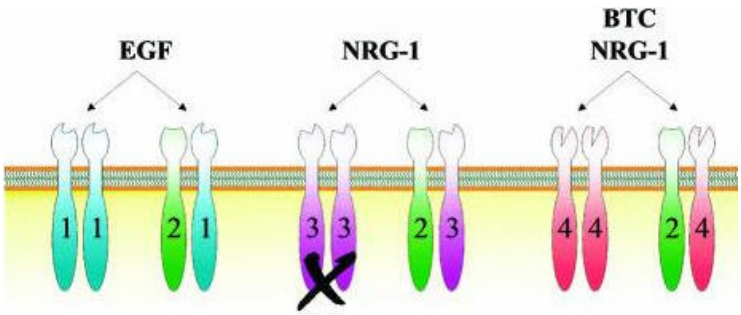


See the text for more details. Note that HER2 lacks known ligands, suggesting its role as a co receptor [58].

However, HER2 lacks a ligand binding site and is considered a co receptor, and in fact the preferred heterodimerization partner for all other members of the HER family. HER3 has

impaired tyrosine kinase activity and therefore needs a heterodimerization partner to be able to participate in signal transduction [58] (fig. 6).

Figure 6: Dimerization partners in the HER family



HER2 is the preferred receptor for dimerization. Note that HER3 does not signal but is capable of ligand binding [58].

Vasculogenesis (embryonic vascular development) and angiogenesis (formation of new blood vessels from pre-existing vasculature) are initiated by ligands in the vascular endothelial growth factor (VEGF) family and receptors belonging to the RTK superfamily; vascular endothelial growth factor receptors 1-3 (VEGFR 1-3). VEGFR1 is a positive regulator of monocyte and macrophage migration. VEGFR2 and VEGFR3 regulate vascular endothelial cells and lymphatic-endothelial cells respectively.

The ligands belonging to the VEGF family are VEGFA, B, C, D, E and the placental growth factor (PlGF). VEGFA, B and PlGF bind to VEGFR1; VEGFA and E bind to VEGFR2 and VEGFC and D bind to VEGFR3. The VEGFR RTKs share most of the signalling mechanisms and pathways with those of the EGFR RTKs, but are in addition able to transduce signals crucial for vascular formation and permeability [60, 61].

Downstream signalling pathways include the mitogen activated protein kinase (MAPK) pathway, which will be emphasized here, the phospho inositide 3-kinase (PI3K) pathway and the phospholipase C γ/protein kinase C (PLC γ/PKC) pathway. The MAPK pathway involves the proto-oncogene Raf-1, a member of the serine-threonine kinase family, acting as a MAPKKK (MAP kinase kinase kinase). MAPKKK phosphorylates and activates the MAP kinase kinase MEK ½, in turn phosphorylating the MAP kinase extracellular signal regulated kinase, ERK. ERK dimerizes upon double phosphorylation and translocates into the nucleus, inducing early response genes such as c-fos, c-jun and c-myc [62] (fig. 3 and 7).
EGFR signalling mediates Cx43 phosphorylation/closure by a mechanism dependent on the MAP kinase ERK5 [63].

1.6 Molecular targeting of EGFR and VEGFR

EGFR is over expressed in 40-60 % of GBMs, and 40-50 % of these tumours contain an amplification of the constitutively active truncated mutant EGFRvIII. Thus, targeting the EGFR signalling pathway seems to be a rational therapeutic approach [64-68].
Preclinical data encourage clinical evaluation of antibodies targeting the extracellular domain of the EGFR [69], and promising clinical outcome in the treatment of solid tumours have been described, especially in combinations with radiotherapy and conventional cytotoxic agents [70, 71]. As for GBM, early clinical trials are ongoing [72].

Figure 7: VEGFR2 signalling

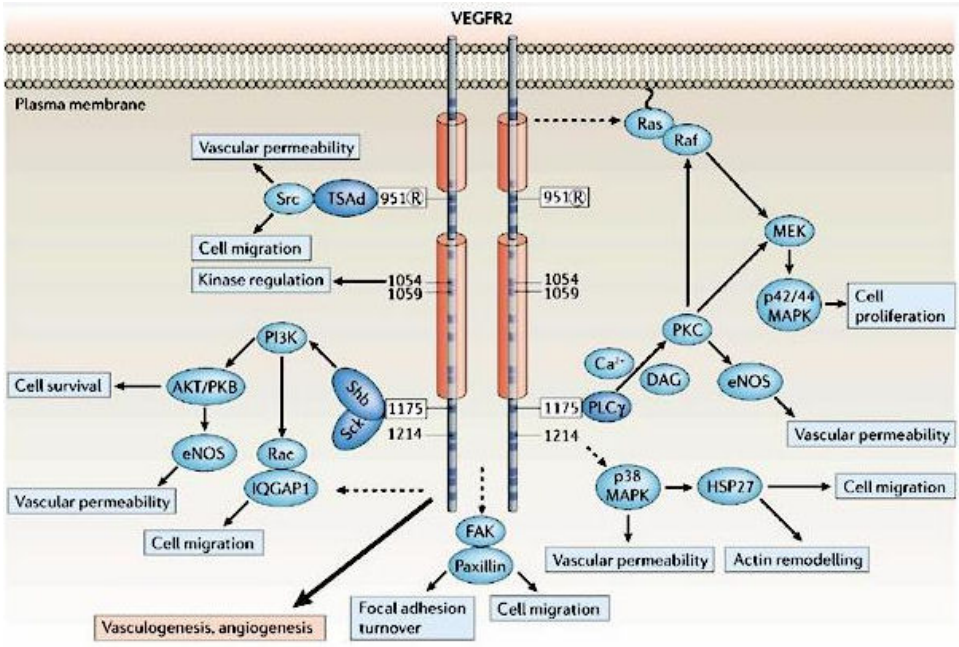


Illustration of the VEGFR2 signalling pathway. Ligand binding initiates an array of mediators and second messengers including the MAP kinases MAPK (mitogen activated protein kinase) and MEK (MAPK and ERK kinase). Dashed arrows indicate unclear mechanisms [60].

Small-molecule EGFR tyrosine kinase inhibitors like gefitinib and erlotinib have attracted much attention in clinical trials, in which monotherapy and combinations with chemotherapy and radiotherapy have been evaluated. Treatment is generally tolerable, with diarrhoea and skin rash as the most common side effects. Unfortunately, these trials have failed to show significant efficacy on time to tumour progression or overall survival in GBM. Moreover, overall survival has been difficult to correlate to EGFR expression [73] [74]. A probably

underestimated aspect is the impact of treatment timing and scheduling on therapeutic efficacy [75]. However, clinical evidence indicates that co-expression of EGFRvIII and intact phosphatase and tensin homolog (PTEN) predict high level of response to EGFR inhibitors [76] and that enhanced EGFR expression in combination with low activity in the PI3K/Akt pathway correlates with better response to EGFR inhibitors [77]. Intriguingly, the latter mechanism could show to be of importance in combination treatment strategies with histone deacetylase (HDAC) inhibitors (section 4.11).

Excessive and abnormal vessel formation, a hallmark in GBM, was realized as early as in the 1940s [78]. Subsequent reports suggested a negative correlation between capillary density and prognosis of the disease [79, 80]. Thus, great efforts targeting the VEGF signalling pathway have been launched. Bevacizumab is a recombinant monoclonal antibody directed towards VEGF, preventing ligand interaction with VEGFR1 and 2. It has shown promising results in solid tumours in combination with chemotherapy, even in recurrent glioma, where a high radiographic response rate has been demonstrated. However, there is still some uncertainty whether the relatively high response translates into prolongation of survival [7, 81].

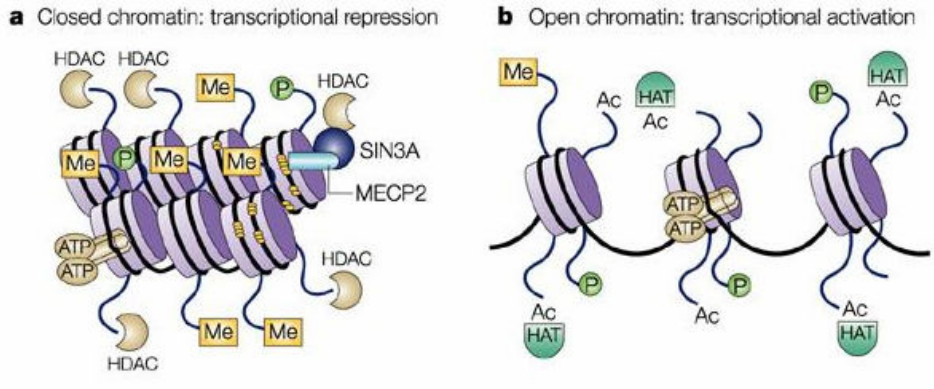
The small-molecule receptor tyrosine kinase inhibitor (RTKI) vandetanib targets EGFR similar to gefitinib, but also inhibits the tyrosine kinases of VEGFR2, 3 and RET [82, 83]. The pharmacokinetic profile supports a once-daily oral dosing regimen with mild side effects similar to those of gefitinib [84, 85]. As for glioma the drug has induced tumour regression, both as monotherapy and in combination with radiotherapy *in vitro* and *in vivo*, in animal models [86-88]. A number of clinical trials for malignant glioma with vandetanib in combination with temozolomide are under way [72].

1.7 Epigenetics and HDAC inhibitors

The strive to unravel the mechanisms contributing to the exceptional plasticity and adaptation potential of tumours inspired Feinberg and Vogelstein in the 1980s, demonstrating in their pioneering work that a substantial proportion of CpG islands which were methylated in normal tissues were unmethylated in cancer cells [89]. These results contributed to the foundation of epigenetics, which explain the ability of different cell types to share the same DNA sequence, while maintaining their cell-type specific characteristics during cell divisions. Besides DNA methylation, epigenetics also include other mechanisms such as histone modifications, which are crucial for DNA replication timing and transcriptional permissiveness. So far, epigenetics has been in the shadows of cancer genetics. But the area is emerging, and the two disciplines are fusing, to diversify our understanding of gene regulation, initiation and progression of cancer [90-93].

Acetylation of lysine residues within histone tails is associated with chromatin remodelling and, usually, transcriptional activation [94]. The acetylation status of core histones is controlled by two families of enzymes; the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). HDAC inhibitors are able to maintain chromatin in an acetylated form, in which the acetyl groups neutralize the positive charge of lysines and thereby their electrostatic interactions with the negatively charged DNA phosphate backbone. This allows a more open chromatin conformation, providing accessibility to the transcription apparatus [95, 96] (fig. 8).

Figure 8: Transcriptional regulation by chromatin remodelling.



DNA wrapped around nucleosomes, consisting of histone octamers. Two functionally diverse chromatin configurations are illustrated in a. and b.

a. DNA methylation and hypoacetylation leads to a condensed and transcriptionally silent chromatin.

b. Histone acetylation and demethylation relaxes the chromatin, initiating the transcriptional apparatus [95].

HDAC inhibitors are potent inducers of differentiation and apoptosis by intervention with a wide array of intracellular pathways (fig. 9). Transcriptional activation of target genes by HDAC inhibitors includes the CDKN1A gene encoding p21 $^{WAF/CIP1}$, mainly in a p53 independent manner [97, 98], as well as members of the INK4 family, for example p16. p21 negatively modulates cell cycle progression in G1, S and G2 by binding to cyclin-dependent kinases (Cdk) and their respective cyclins. Additionally, p21 halts DNA replication by interaction with the proliferating cell nuclear antigen (PCNA), a DNA polymerase implicated in DNA replication and repair, leading to G1 arrest. Many HDAC inhibitors also down regulate cyclin D and A, leading to hypophosphorylation of pocket proteins, like the

retinoblastoma protein (RB), with a subsequent arrest in S-phase of the cell cycle [95, 96, 99-103]. Moreover, HDAC inhibitors have been shown to up regulate the expression of pro apoptotic proteins, for example Bad, Bax, Bim, Bmf and Bak, as well as down regulation of antiapoptotic proteins such as Bcl-2 and Bcl-xl [68, 104-107]. Angiogenesis is inhibited by repression of proangiogenic factors such as hypoxia inducible factors (HIF) [108], which function as transcription factors for angiogenic genes [109], VEGF and VEGFR [110, 111]. Moreover, expression of some antiangiogenic factors are induced, such as semaphorin III (a VEGF competitor), p53 and von Hippel-Lindau (VHL) [110, 112].

Importantly, recent evidence suggest that the activity of HDAC inhibitors is not confined solely to histone modulation, but also to nonhistone proteins including transcription factors, signal transduction mediators, a microtubule component and a molecular chaperone. Well-known nonhistone substrates include p300, p53, Smad7, Stat3, NF-κB, α-tubulin and Hsp90 (reviewed in [113]). As previously mentioned, p53 is also targeted by HDAC inhibitors on the gene expression level by histone acetylation, which accelerates transcription of the protein [112].

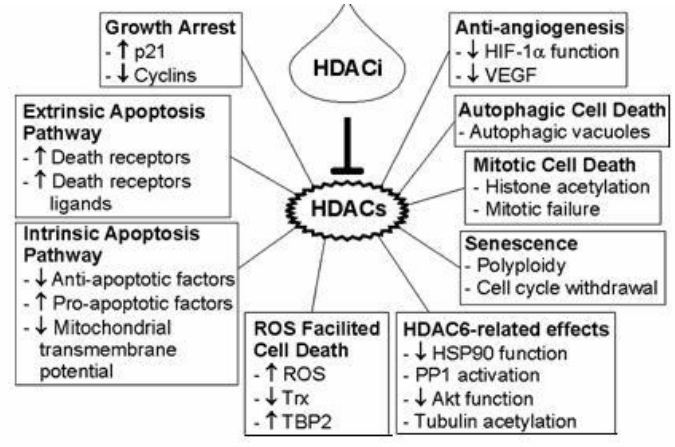
Intriguingly, only a minor proportion of genes are affected by HDAC inhibitors. Early differential display experiments showed alterations in gene expression in 2% of the examined genes, but a more recent assessment based on cDNA array methods was in the range of 7-10% [114-117]. In addition, normal cells are relatively resistant to HDAC inhibitors compared to transformed cells [118, 119].

Accumulation of reactive oxygen species (ROS) in transformed cells, but not to the same extent in normal cells treated with HDAC inhibitors, could give us a clue to tumour specificity associated with HDAC inhibitors. A plausible mechanism is a more efficient inactivation and down regulation of the ROS antioxidant scavenger thioredoxin (Trx) by Trx binding protein 2 (TBP2) in transformed cells in comparison to normal cells in the presence

of HDAC inhibitors [119-121]. Another mechanism explaining differential sensitivity could be a loss of G2 checkpoint in many cancer cells, making them vulnerable to apoptotic stimuli by HDAC inhibitors [103].

4-phenylbutyrate (4-PB), an aromatic fatty acid and an HDAC inhibitor can reach biologically active serum levels with oral administration [122, 123] and has shown activity in patients with GBM with mild side effects [122, 124].

Figure 9: Signalling pathways activated by HDAC inhibitors [107].



1.8 The CNS cytoskeleton

The three main types of cytoskeletal structures are the intermediate filaments, microtubules and the actin filaments. The 50 kDa glial fibrillary acidic protein (GFAP) belongs to the intermediate filament (IF) family, which contributes to the cytoskeleton and the nuclear envelope of most vertebrate cells. GFAP is the most common intermediate filament protein in mature astrocytes and was initially isolated by Eng at al. in the late 1960s from patients with multiple sclerosis [125-127]. Our view of the IFs as a rather stable structure compared to actin

filaments and microtubules has now been revised. Like other IF proteins, GFAP comprises an aminoterminal head domain, a central rod domain and a carboxyterminal head domain [128]. The assembly and disassembly of GFAP is regulated by its phosphorylation status. Protein phosphorylation affects the dynamic equilibrium of GFAP in the cell and breaks down the cytoskeletal network [129]. Phosphorylation occurs on specific serine and threonine residues of the head domain of GFAP by several kinases, such as protein kinase A (PKA), protein kinase C (PKC), Ca^{2+}/calmodulin dependent protein kinase II (CaMKII), cdc2 kinase and Rho kinase, which leads to disassembly of the filament structure *in vitro* [130, 131]. Reorganization of the cytoskeleton is one of the fundamental mechanisms in the mitotic apparatus, especially in cytokinesis, in which the cytoplasm of a mitotic cell is separated into two daughter cells [132].

Increased expression of GFAP accompanies the differentiation of astroglia, while glial immaturity, as in tumour transformation, decreases expression. This property has made GFAP a reliable marker of assessing the differentiation status of tumours of astrocytic origin. Interestingly, increased concentrations of non-phosphorylated GFAP have been shown to suppress the growth of astrocytomas and reverse the neoplastic phenotype both *in vitro* and *in vivo* [133].

Early precursor cells of glial origin express the 52 kDa IF protein vimentin. During the differentiation process, the expression declines and is replaced by an increasing GFAP expression [134]. GBMs can express both of the IFs [135]. Furthermore, the presence of vimentin is a prerequisite for proper assembly of the nestin IF [136]. Nestin plays an important role in the developing CNS and is expressed in CNS stem cells. During the formation of the adult brain, expression of nestin is gradually decreased and is only rarely present among endothelial and subependymal cells. Moreover, there seems to be a positive correlation between nestin expression and malignancy grade in glial tumours, with the highest

expression in GBMs [137, 138]. There are also pieces of clinical evidence justifying nestin as a diagnostic and prognostic marker in brain tumours [139, 140].

S100-proteins are Ca^{2+}-binding polypeptides with a proposed role in tumour progression, and also considered as a potential tumour marker, with an expression rate according to tumour grading [141, 142]. A pilot study by Vos and co-workers, found a negative correlation between serum levels of S100B and survival in glioma patients [143]. Subtypes of the S100 family stimulate angiogenesis and correlate with neovascularization in astrocytic gliomas [144] and, moreover, stimulate the cellular migration rate by reorganization of actin filaments [145].

Microtubules are long hollow cylinders, which are polymers of α/β tubulin heterodimers. They exhibit a wide array of vital functions in the cell such as organization of the mitotic spindle, cilia and bundles inside neurons. β-tubulin III, one of the tubulin isotypes, is almost exclusively neuron specific. It can be used as a marker for neuronal differentiation in embryonic stem cells [146, 147].

2. AIMS OF THE THESIS

- To further investigate the role for gap junctions in suicide gene therapy of GBM.

- To investigate whether enhancement of gap junction communication can be accomplished and its relevance for gene therapy utilizing neural progenitor cells.

- To initiate the investigation of Cx43 as an actor in signal transduction inhibition.

- To evaluate combination treatments of an HDAC inhibitor and RTKIs with correlation to Cx43 expression.

3. MATERIALS AND METHODS

3.1 Tissue processing and cell culture

3.1.1 Tissue processing and primary cultures of GBM cells (I, II)

Tumour tissue specimens were obtained from patients undergoing open surgical resection of a GBM (WHO Grade IV) according to peroperative diagnosis on cryostat sections, subsequently confirmed with conventional histopathological examination. Specimens of 1–5 mm^3, acquired with mild mechanic dissociation, were applied on laminin and polyornithine coated 3 cm petri dishes before adding 200-300 µl of culture medium (DMEM/F12 medium supplemented with 10% FCS). After allowing the tissue to settle for 24 hours an additional 1 ml of culture medium was added. Thereafter medium was changed every 48 hours. After 2-3 weeks a radial outgrowth of tumour cells could be visualized with phase contrast microscopy.

3.1.2 Development of the NGC-407 cell line (III)

The NGC-407 cells used in this study were developed by NsGene A/S, Denmark, in collaboration with the University of Lund, Sweden. The cells originate from the ventral mesencephalon of a 7-week-old human embryo and were immortalized by transfection with a retroviral vector containing the v-myc oncogene (with permission of the Ethics committee in Lund).

3.1.3 Cell culture of NGC-407, HNSC.100, U87MG and U343MGa (III, IV)

NGC-407 (section 3.1.2) and HNSC.100 (a nestin-positive, human neural stem cell line perpetuated by v-myc) cells were plated on poly-l-lysine coated petri dishes and cultured as adherent cells. Culture medium was supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Cells were differentiated by withdrawing mitogens (bFGF and EGF) from culture medium. The frequently employed and well characterized

U87MG and U343MGa cells were also used [148, 149] as well as GB29 cells, which were established as tissue explants at our laboratory (section 3.1.1).

3.2 Immunocytochemistry (I-IV)

Cells were washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PF) in phosphate buffer for 10 min. After washing with PBS they were incubated with primary antibodies. Secondary antibodies conjugated with fluorescein isothiocyanate (FITC) or cyanine 3 (Cy3) were applied on cells for 1 h at room temperature. The procedure was completed with rinsing with PBS. Bound antibodies were visualized with epifluorescence microscopy. Primary antibodies detecting GFAP, S-100, Cx43, nestin, vimentin and anti-β-tubulin III were used.

3.3 Western blotting (I-IV)

Protein concentrations were quantified using the micro BSA protein assay. Cell lysates were mixed with loading buffer before loading into 10% polyacrylamide gels, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), and electrically transferred to nitrocellulose membranes with a transblot apparatus. Primary antibody in combination with the enhanced chemiluminescence (ECL) detection system was used.

3.4 Assessment of gap junction communication and its inhibition

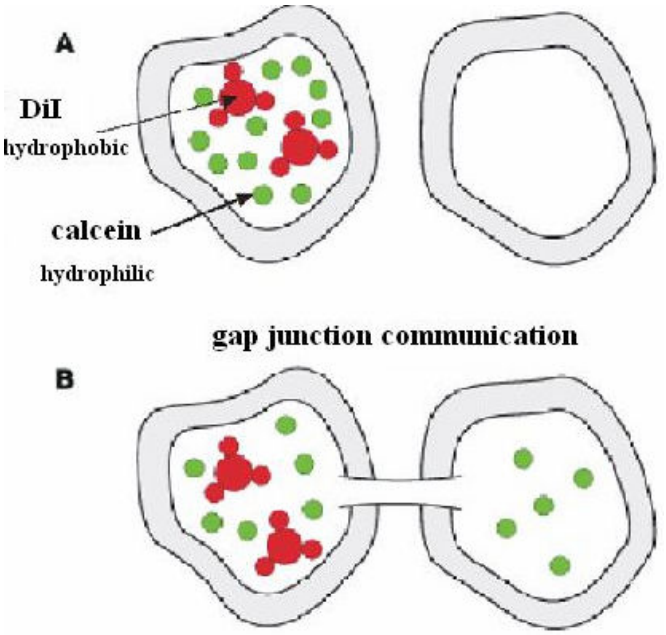
3.4.1 Assessment of gap junction communication by calcein and DiI (I-III)

In this work we have utilized a pre-loading method evaluating GJC established by Goldberg and co-workers using the fluorescent probes calcein and dioctadecyl tetramethylindocarbocyanine perchlorate (DiI) [150], adapting it to our experiments.

When calcein is introduced to the cell, the acetomethoxy group of calcein is cut off by intracellular esterases. After chelating with calcium a strong green fluorescence evolves. This fluorescing chelate complex gets trapped inside the cell, but is able to diffuse to its neighbours through gap junctions. The membrane staining fluorescent probe (DiI) is confined to the primary stained cell due to its lipophilicity.

When double staining of calcein and DiI was performed, stained cells were trypsinized and plated on top of a population of unlabelled cells. While calcein was able to spread to the unlabelled population, DiI was not. This made it possible to distinguish the primary stained cell population, due to its DiI stain, from the previously unlabelled population. By correlating the number of double stained cells (calcein+DiI) to mono-stained cells (calcein only) an assessment of calcein transfer could be made as a measure of GJC (fig. 10).

Figure 10: A preloading method using DiI and calcein



a. *Double staining with the fluorescent probes DiI and calcein. DiI is lipophilic and calcein is water soluble, which facilitates diffusion via gap junctions.*

b. *After co-plating stained cells with a population of unlabelled cells, calcein spreads to neighbouring cells, while DiI is confined to primary stained cells. Intercellular diffusion of calcein is gap junction dependent.*

3.4.2 Fluorescent dye transfer studied in glioma cells (I-III)

Primary glioma cells were cultured on 3-cm petri dishes and labelled with calcein and DiI diluted in serum-free medium and incubated for 30 min at 37°C. The cells were washed with PBS, trypsinized, and suspended in DMEM/F12-supplemented medium including 10% FCS, followed by centrifugation and resuspension in fresh culture medium. The procedure was repeated twice. Following the second resuspension, the labelled cells were transferred to a dish with unlabelled cells, allowing the labelled cells to settle. The intercellular transfer of water soluble calcein was evaluated and correlated to lipophilic DiI staining as a measure of GJC (fig. 6).

3.4.3 Inhibition of gap junctions by 18 α glycyrrhetinic acid (AGA) (I)

A derivative of one of the primary components of liquorice root glycyrrhiza radix, 18 α glycyrrhetinic acid (AGA), have proved to be a reversible inhibitor of gap junctions [151] [152]. AGA was added to cell culture medium with the intention to study the impact of gap junction inhibition on calcein transfer (section 3.4.2) and of the bystander effect in the HSV-tk/GCV system (section 3.6).

3.5 Cloning of plasmids (I)

The cloning of pTK-SIRES-Neo was made by inserting the tk gene as a *Bam*HI fragment from pVP22-tk with three stop codons in all reading frames, into pIRES-Neo containing the cytomegalovirus major immediate early promoter/enhancer.

The cloning of pBudCE4.1 EGFP/tk sense/antisense was made by cutting pEGFP-C3 by *Sca*I and *Bam*HI. After blunting the restriction sites the religated vector was cut by *Nhe*I and *Hpa*I and the insert transferred into pcDNA3.1zeo vector digested by *Nhe*I/*Eco*RV. In a final step the blunted *Nhe*I/*Hin*dIII fragment from this plasmid was cloned into the *Xho*I site of pBudCE4.1. The tk gene was isolated by *Bam*HI digestion from pSIRES-tk and cloned either in sense or antisense orientation into the *Sca*I site of the pBudCE4.1 vector carrying EGFP. The identities of the clones were confirmed by sequencing.

3.6 The HSV-tk/GCV system and evaluation of cell death (I)

Glioblastoma cells were transfected with the pBudCE4.1EGFP/tk vector, with the tk gene expressed in sense or antisense orientation. After transfection, sense- or antisense-transfected cells were treated either with AGA or with the equivalent volume of DMSO prior to GCV treatment. After 96 hours a quantitative cell viability test, MTT, was used. For the qualitative analysis of cell toxicity, phase contrast microscopy was employed to evaluate cell death in cells transfected with the plasmid pTK-SIRES-Neo, and treatment time was extended to a maximum of 10 days.

3.7 Fluorescent dye transfer in NGC-407 and glioma cells (III)

Both donor and recipient NGC-407 cells were seeded on poly-l-lysine coated 3 cm-petri dishes and treated with 0.5 mM 4-PB for 120 h, either in the presence or absence of growth factors. Donor cells were then incubated for 20 min at 37 °C with DiI and calcein in serum

free medium. These double labelled cells were rinsed with PBS, trypsinized and added to a subconfluent monolayer of unlabelled NGC-407, U87MG or U343MGa cells at a ratio of 1:50 (labelled: unlabelled). Unlabelled U87MG and U343MGa cells were treated with 2 mM 4-PB for 120 h prior to addition of labelled NGC-407 cells, which were treated with 0.5 mM 4-PB. Fluorescent dye transfer was evaluated as described in section 3.4.2.

4. RESULTS AND DISCUSSION (I-IV)

Although a concomitant approach of temozolomide and radiotherapy in the treatment of GBM has demonstrated clear clinical benefit, prolongation of median survival was only a few months [6]. New treatment paradigms are therefore of urgent need.

The two novel therapeutic strategies introduced in this study are HSV-tk mediated gene therapy utilizing NPCs in conjunction with the HDAC inhibitor 4-PB (I-III) and combination treatment with HDAC inhibitors and RTKIs (IV). Here we propose a model in which the dual mechanisms of Cx43, serving as a gap junction protein as well as a tumour suppressor, are exploited, while raising arguments for Cx43 as a target in glioma therapy.

In the gene therapy approach we have utilized Cx43 as a gap junction component and as a mediator for the bystander effect. In the combination model we have employed the tumour suppressing effect of Cx43 in an attempt to explain the therapeutic effects of HDAC inhibitors and RTKIs.

4.1 Establishment and characterization of GBM cultures (I)

The heterogeneity of tumour cells in GBM confronts us with a complicated and intricate task, when we try to transfer the complexity of the tumour to the petri dish. Therefore, it is realized that by studying homogeneous cell lines, problems arise in the attempt of directly interpreting results without the risk of oversimplification [153]. Various models of explant cultures have

been developed, and it has been shown that surgically removed glioma specimens can be cultured as explants [154] or spheroids [155]. Thus, it may be possible to somewhat increase the heterogeneity of the cell population. However, it is worth emphasizing that even these efforts will result in an adaptation of the cell population to culture conditions, and increasingly so after each cell passage.

In order to minimize disruption of tissue architecture, tumour specimens were subjected only to mild mechanical dissociation prior to plating. Once adherent to the substrate, tumour cells compliant to the culture conditions proliferated, migrated and expanded. Despite optimized culture medium and substrate conditions, only about half of the surgical tissue samples yielded a successful primary culture.

Repeated immunocytochemistry (ICC) with GFAP and S100 verified the glial characteristics of the cells. In addition, cells showed vimentin and nestin immunoreactivity, which indicated an immature phenotype. Moreover, most of the cell cultures studied expressed Cx43 at various levels detected by ICC and Western blotting.

4.2 AGA effects on GJC (I)

Western blotting showed that both the phosphorylated and the non-phosphorylated form of Cx43 were down regulated in the presence of the gap junction inhibitor AGA in this study, revealing a feasible mechanism, probably among others [152], of the AGA effects.

When GBM cells were stained with the calcein/DiI preloading method (section 3.4.1), a significant intercellular calcein transfer was evident, which indicates the presence of functional gap junction coupling. Performing this experiment in the presence of AGA, calcein diffusion between cells was markedly reduced compared to control cells, indicating gap junction inhibition by AGA.

4.3 The role of GJC in the HSV-tk/GCV system (I)

The bottleneck of gene therapy is insufficient delivery of the therapeutic gene or gene product to tumour cells [156]. Here we investigated the role for gap junctions as a mediator of the bystander effect in a GBM model.

When GBM cells were transfected with a plasmid encoding HSV-tk and were treated with GCV, a majority of cells was killed after 10 days. Interestingly, this cell killing is manyfold higher than the estimated transfection efficiency of only about 5-10%. This amplification of cytotoxicity is due to the so called bystander effect, in which suicide gene positive cells spread toxic metabolites to their neighbours. By using the specific gap junction inhibitor AGA it was possible to markedly hamper cell killing, suggesting a gap junction dependent bystander effect. In an attempt to extrapolate our findings to the clinical situation, it is noteworthy that our monolayer cultured cells are insufficient to reflect the heterogeneity and three-dimensional structure of the original tumour. However, unambiguously, gap junctions seem to play an important role in glioma cells by their clear expression of Cx43 and by their ability to form gap junctions. Thus, gap junctions are probably one of the key issues to address conferring gene therapy the status of a theraputic option in GBM.

4.4 Differentiation of glioma cells by HDAC inhibitors (II)

While HDACs are able to keep chromatin acetylation at a low level, their inhibitors shift the acetylation balance, turning chromatin into a transcriptionally active state. The HDAC inhibitor family consists of a very heterogeneous group of substances with diverse chemical and biological properties.

In this work we have mainly focused on the small fatty acid 4-PB. The main reasons for the choice are its tolerability, low molecular weight and the possibility of achieving therapeutic serum and CSF levels with oral administration [122, 123]. Furthermore, promising effects

have been documented in early clinical trials of GBM [122, 124, 157]. In our glioma model, we first investigated the effects on morphology and cell viability with the HDAC inhibitors 4-PB, splitomicin, trichostatin A and valproic acid. The most obvious morphologic effect in our glioma model was elongation of cell processes and a more bipolar phenotype, consistent with a differentiation effect. ICC interrogating GFAP showed an increased perinuclear distribution of the IF. Additionally, two out of three cultures displayed an increased general GFAP expression observed by Western blotting, which is in line with a progression to a more differentiated phenotype with rearrangements of IFs.

4.5 HDAC inhibitors increase Cx43 expression and functional GJC (II)

ICC and Western blotting detected a 4-PB induced increase in Cx43 expression levels and, additionally, Cx43 redistribution to cytoplasmatic processes. The explanation for its relocalization to the cell periphery could be adaptation to a more differentiated state, potentiating intercellular contacts. Western blotting revealed that both phosphorylated and non-phosphorylated isoforms were up regulated. An increase of the non-phosphorylated isoform, based on our knowledge of channel regulation [26, 29, 158], suggests an increase of open gap junctions. With the intention to investigate whether this enhancement of Cx43 expression was accompanied by improved intercellular contacts, we utilized the previously described DiI/calcein preloading method. It was modified by pre treating cells with 4-PB. As expected, an increase in calcein diffusion could be seen, assessed as the amount of secondary calcein stained cells.

Since these results were published, a number of other researchers have confirmed our observations of Cx43 and GJC induction by HDAC inhibitors in both non malignant cells and tumour cells of the prostate and nasopharynx [159-161]. Some authors explain Cx43 up regulation by HDAC inhibitors as a consequence of hyperacetylation of histones on the Cx43

promoter [159, 160], while one group was unable to find a correlation between histone acetylation and Cx43 induction, assuming that the reason could be an acceleration of Cx43 translation or an indirect effect of regulation of degradation of the protein by HDAC inhibitors[161].

Taken together, our findings, in accordance with other groups, demonstrate a 4-PB induced up regulation and redistribution of Cx43 with a parallel increase in the assembly of functional gap junctions. Thus, it seems rational to conclude that 4-PB, as well as other HDAC inhibitors, could play an essential role in facilitating the bystander effect, thereby expanding the therapeutic range for gene therapy.

4.6 Mitogen withdrawal induces differentiation and Cx43 down regulation in NPCs (III)

Diffuse infiltrative growth and migration are hallmarks of malignant glioma, considerably complicating the definition of tumour borders in the clinical situation. In order to reach the outskirts of the tumour, we would need a strategy able to track down every single tumour cell and target it with a specific treatment. A promising paradigm involves neural progenitor cells (NPCs), which show extensive tropism towards glioma cells *in vitro* and *in vivo* [52, 53, 56, 57, 162].

In this work we have used two different neural progenitor cell lines, the NGC-407 originating from midbrain, and HNSC.100 from forebrain. NGC-407, a non-committed progenitor cell line, is especially valuable in that it is characterized, still preserved and approved by an ethics committee, and thus possible to use in future clinical applications.

In culture conditions, when mitogens were included, proliferation was promoted and nestin was expressed. On withdrawal of mitogens two different cell lineages appeared; one expressing GFAP and one expressing β-tubulin III, with no or minimal overlap between

expression patterns. In parallel, nestin expression was decreased. The β-tubulin III expressing cells displayed a delicate network of long and slender processes, contributing to about 10 % of the cell population. The rest of the population, which was GFAP positive, consisted of elongated, multipolar cells.

The lineages were characterized as cells with neuronal and astrocytic phenotype, respectively. The conclusion drawn from these data is that mitogen withdrawal from the NGC-407 cells triggers a bilinear differentiation process towards either glial or neuronal characteristics.

Gap junctions have shown to play a key role in the maintenance of pluripotency, proliferation and migration in embryonic stem cells and neural progenitors [163, 164]. These results are in accordance with our data, in which mitogen deficiency induced differentiation and down regulated Cx43 levels.

4.7 4-PB increases Cx43 expression in NPCs (III)

We hypothesized that analogous to the 4-PB up regulating effect on Cx43 and GJC in glioma cells a similar effect in NPCs would be expected. To this end, Cx43 expression and two phosphorylation isoforms were analyzed in both proliferating and differentiating NPCs with ICC and Western blotting, in the presence or absence of 4-PB.

A comparison between proliferating and differentiating cells showed that the differentiation process, in contrast to glioma cells, down regulates levels of Cx43. However, the phosphorylated isoforms, P-Ser279/282 and P-Ser368, were also down regulated. Importantly, 4-PB increased Cx43 expression levels in both proliferating and differentiating cells. At the same time a reduction of the relative expression rate of phosphorylated isoforms was found, with the exception for Ser368 (phosphorylated by PKC) in differentiating cells, but not in proliferating cells. Reports addressing this seemingly contradictory issue suggest differential

gating responses at this specific phosphorylation site depending on maturation status of cells [165, 166].

In conclusion our data show that 4-PB up regulates the gap junction protein Cx43 in NPCs, but also show a tendency towards a more dephosphorylated Cx43 state, which may be consistent with a more open gap junction conformation.

4.8 4-PB enhances GJC between NPCs and glioma cells (III)

The next issue to address was whether NPC-glioma cell coupling could be promoted by 4-PB, based on our knowledge of 4-PB mediated up regulation of Cx43 in glioma cells and in NPCs (II, III, section 4.5 and 4.7).

To this end, the previously described DiI/calcein preloading method was performed. A clear increase of calcein diffusion could be visualized after 4-PB treatment in both proliferating and differentiating NPCs, as well as between NPCs and either of the two glioma cell lines U87MG and U343MGa, which is in line with our hypothesis of 4-PB induced enhancement of GJC.

As previously discussed (section 1.4), there is substantial evidence that NPCs have the propensity of tracking down and targeting GBM cells and efficiently delivering tumour toxic gene products [54, 162, 167-170]. Additionally, we demonstrate a mechanism which could potentiate transfer of these antitumoural products, possibly improving the efficacy of suicide gene therapy.

4.9 Morphological and cytotoxic effects by 4-PB, gefitinib and vandetanib (IV)

With the intention to mimic the clinical situation as closely as possible, drug concentrations in cell culture medium were in the same range as therapeutic plasma concentrations in clinical

trials [84, 122, 171]. Additionally, drug exposure time was relatively long, with a maximum of 7 days, with replenishment of fresh drug containing culture medium every 24 hours. Phase contrast microscopy and a cell viability test, MTT, were used to study morphological and cytotoxic characteristics of glioma cells exposed to gefitinib and vandetanib. Cells subjected to gefitinib in rising concentrations of 1-10 μM gradually displayed nuclear granulation, which was interpreted as signs of apoptosis, while cell density appeared to decline. However, the nuclear-cytosolic ratio seemed to be preserved. In contrast, vandetanib in 2-6 μM induced a more elongated and bipolar phenotype with a low nuclear-cytosolic ratio. Gefitinib caused rather abrupt detachment of cellular monolayers in concentrations exceeding 30 μM, while the corresponding vandetanib concentration was in the range of 4-6 μM. Thus, vandetanib showed a potency 5-10 fold, depending on cell line, the one for gefitinib. Treatment with 2 mM 4-PB alone displayed elongation of cell processes and a clear reduction in the nuclear-cytosolic ratio, consistent with results previously presented (II), resembling the phenotype observed in vandetanib treated cells. Addition of 4-PB to gefitinib or vandetanib treated cells lowered the concentrations required for cell killing effects of either of the RTKIs. MTT test confirmed the visual impression obtained by phase contrast microscopy.

4.10 Gefitinib and vandetanib elevate Cx43 expression levels (IV)

Recent years of cancer research have resulted in a shift in treatment paradigm from conventional cytostatic agents to substances targeting specific molecular pathways. This is mainly due to the accelerating knowledge and understanding of biological mechanisms, which differs the cancer cell from its normal counterpart. The overall aim of this development is to expand the therapeutic ratio by improving efficacy of tumour cell reduction, while minimizing the risk for side effects.

Gefitinib and vandetanib, products from this development, both act as RTKIs of the EGFR pathway. Additionally, vandetanib also targets VEGFR and RET signalling [82, 83, 172]. As mentioned (section 4.5 and 4.7) we have, for the first time, reported up regulation of Cx43 by an HDAC inhibitor (II), subsequently confirmed by other researchers [159-161]. Furthermore, we have also demonstrated Cx43 up regulation in association with RTKIs (IV). Western blotting detected a marked increase in Cx43 expression levels by gefitinib and vandetanib. ICC confirmed these data, but also indicated a redistribution of the protein to cytoplasmatic vesicles and processes, in the vicinity of intercellular contact zones. This expression pattern is very similar to the one exhibited in cells treated with 4-PB, as previously observed (II). Leite and Rivedal reported that EGF accelerates ubiquitination, internalization and proteasomal degradation of Cx43 via a MAPK dependent pathway [30]. This could explain why RTKIs, inhibiting this pathway, contribute to elevation of Cx43 expression. Interestingly, in conjunction with treatment with gefitinib and vandetanib, a distinct band in the 20-25 kDa region appeared, probably consisting of the Cx43 carboxy terminal (CT-Cx43) [173, 174]. An emerging view of the CT-Cx43 as a tumour suppressor in its own right, and even with equal efficiency as wild type Cx43, is presently under scrutiny [11, 22, 24]. Some of the proposed mechanisms for CT-Cx43 induced tumour suppression are degradation of the human F-box protein Skp-2, synergism with cadherins in regulating β-catenin translocation to the nucleus, and anchoring zonula occludens-1 (ZO-1) scaffolding protein to the plasma membrane [11, 22, 68, 173, 174]. CT-Cx43 induction has previously been associated with exposure to the cytostatic agent cyclosporin [174], but to our knowledge not previously described in conjunction with RTKIs.

4.11 Cooperative effects with 4-PB and receptor tyrosine kinase inhibitors (IV)

To address the issue of tumour cell heterogeneity, a hallmark in GBM and probably of vital importance for therapeutic resistance, we here introduce an *in vitro* combination therapy approach. The rational is to evaluate cooperative effects of the epigenomic modulator 4-PB in combination with molecular targeting by tyrosine kinase inhibitors, intervening into a multitude of tumour specific signalling mechanisms using drugs with few side effects [69, 84, 107, 122, 124, 171, 175, 176]. Interestingly, recent data support that HDAC inhibitors dephosphorylate Akt by disrupting HDAC-protein phosphatase 1 (PP1) complexes, acting as a nonhistone substrate, in for example U87MG cells [113, 177]. This provides a rational for combination therapy with RTKIs regulating the Akt pathway, such as gefitinib and vandetanib.

We combined 4-PB and either of the RTKIs gefitinib and vandetanib in clinically relevant concentrations in glioma cell cultures. Additive, or even synergistic effects among different glioma cell lines were observed with microscopic examination and the quantitative cell viability test MTT.

The suggestion of Cx43 as a potential anti-oncogenic target has recently been raised [178]. Interestingly, 4-PB, gefitinib and vandetanib are inducers of Cx43, and additionally, gefitinib and vandetanib are both able to promote expression of a protein entity interpreted as the CT-Cx43. It is thus reasonable to assume that tumour suppression via the Cx43 pathway could play an important role in the downstream effects of RTKIs.

5. CONCLUSIONS

- Gap junctions are important for the bystander effect in suicide gene therapy utilizing the HSV-tk/GCV system in glioma cells (I).

- The HDAC inhibitor 4-PB up regulates the tumour suppressor Cx43 in glioma cells and in neural progenitor cells (NPCs) (II, III).

- 4-PB enhances gap junction communication in glioma cells, NPCs as well as between glioma cells and NPCs, which could prove beneficial for improving delivery of cytotoxic gene products in gene therapy (II, III).

- The RTKIs gefitinib and vandetanib enhance Cx43 expression and probably also the carboxy terminal of Cx43 (CT-Cx43), which could show to be an important mechanism of action for RTKIs (IV).

- Cooperative cytotoxic effects were seen in combination treatments with 4-PB and either of the drugs gefitinib and vandetanib, which could prove beneficial in the development of new therapeutic strategies for GBM (IV).

6. GENERAL CONCLUSIONS

The gap junction complex and its building blocks, the connexins, are of vital importance for cellular homeostasis, coordination and response to external stimuli [8, 12]. Moreover, with accumulating evidence the connexins have acquired the status of tumour suppressors [11]. Targeting connexins thus seems an appealing therapeutic approach [178]. This work has focused on the most ubiquitous connexin, Cx43.

The main theme has been the study of Cx43 from two different perspectives, as a component of the gap junction complex (I-III) and its property as a tumour suppressor protein, and exploiting these characteristics in the development of two different therapeutic strategies (II-IV).

The first strategy involves enhancement of GJC by 4-PB in order to improve transfer of cytotoxic compounds to tumour cells in gene therapy, utilizing neural progenitor cells. Our results indicate up regulation of Cx43 with a concomitant increase of gap junction dependent intercellular dye transfer. These findings suggest the potential of 4-PB as an adjunct in suicide gene therapy (II, III).

Our second strategy is an attempt to utilize the tumour suppressing effect of Cx43. We have demonstrated that the RTKIs gefitinib and vandetanib up regulate Cx43 expression, while inducing a protein in the 20-25 kDa range, interpreted as the carboxy terminal of Cx43 (CT-Cx43) (IV). Gradually more evidence has evolved that may establish CT-Cx43 as an important factor, or even the main contributor, to the Cx43 induced tumour suppressing effect [11, 24, 173].

By combining 4-PB and RTKIs we have observed significant additive cell killing effects (IV). However, if these cooperative effects are dependent on Cx43 regulation is still elusive. Nevertheless, Cx43 appears to be an important candidate target in the downstream effects of RTKIs.

7. FUTURE PERSPECTIVES

7.1 Cx43-a potential tumour marker and target molecule in novel therapies?

Our aim is to continue to investigate the prospects of the tumour suppressor Cx43 as a prognostic indicator and a targeting molecule involved in novel therapies of glioma, by taking advantage of the feasibility to up regulate its expression with drugs with a low toxicity profile, as demonstrated in the present study. We have observed 4-PB induced up regulation of Cx43 and enhancement of gap junction communication in glioma cells and neural progenitor cells as well as an accelerated heterotypic gap junction coupling between the two populations. We have also shown that the RTKIs gefitinib and vandetanib both up regulate Cx43. The potential therapeutic benefit of combining HDAC inhibitors and RTKIs are further substantiated by the observed potentiating cytotoxic effects when combining 4-PB and EGFR-TKIs. Thus, the efficacy of our two proposed novel therapeutic *in vitro* strategies; HDAC inhibition in combination with RTKIs and HDAC inhibition combined with NPCs utilizing the HSV-tk/ GCV system will be further evaluated in future studies.

In this work we have introduced and discussed two novel promising therapeutic strategies for GBM and a tumour suppressor, Cx43, which seems to be involved in their mechanisms of action. Our hope is that this knowledge will contribute to improvement of the prognosis of patients suffering from this highly malignant disease.

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