

The importance of mitochondria in the tumourigenic phenotype: Gliomas as the paradigm (Review)

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Abstract. Cancer arises from the accumulation of nuclear and cytoplasmic abnormalities, a phenomenon allowing for the expression of the tumourigenic phenotype. Gliomas represent the most frequently diagnosed tumours of the central nervous system in adults. Warburg hypothesized the importance of glycolysis in cancer cells, and implicated additional roles of mitochondria in neoplasia. Recent data have shown the importance of mitochondria in the tumourigenic phenotype, in particular, within the apoptotic process. There have been a variety of studies conducted on brain tumours revealing significant alterations of mitochondria within the tumourigenic phenotype. This review describes some of the more recent findings of mitochondria and gliomas, correlating findings to those observed in other cancers. Alterations in mitochondrial DNA copy number and location, as well as dependence of the cancer cell phenotype on mitochondria are emphasised. In addition to its role in apoptosis, the mitochondrion serves as an important element in the tumourigenic phenotype, and clinical approaches targeting this organelle have potential for the development of effective treatment regimens for patients with glioma and other neoplastic diseases.

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1. Introduction

Gliomas represent the most common form of intracranial tumour, comprising about 60% of all primary central nervous system (CNS) neoplasms. Glioblastoma, the most common glioma, particularly in adults, is the most malignant and undifferentiated type, and represents 50-60% of all gliomas diagnosed (1). These tumours have a poor prognosis, with less than 15% of patients surviving 2 years. Lower grade tumours, such as the anaplastic astrocytoma and astrocytoma, occur less frequently; for the former, recurrence, typically at higher grade, is the rule; for the latter, the clinical course is more varied, particularly with specific types of low grade tumours which require only surgery for therapy (e.g. pilocytic astrocytoma, ganglioglioma, neurocytoma) (2). Nevertheless, some fraction of the patients diagnosed with astrocytoma will recur, and similarly to the anaplastic type, at a higher grade. As a result, gliomas continue to be a refractory cancer, where therapeutic options are significantly less than optimal, and additional approaches for treatment are required.

Conceptually, understanding the behaviour of tumour cells by analysis of the phenotype is an important aspect necessary to develop preventative measures in addition to treatment modalities which are both effective and safe for patients with cancer. This 'tumourigenic phenotype' has, in the past, been typically evaluated using approaches primarily addressing nuclear-encoded genes. However, it is clear that the influence of cytoplasmic components of the cell can also play a role in the manifestations of malignancy. As a component of the cytoplasm, the mitochondrion has more recently been investigated as a source of influence on the tumourigenic phenotype. There has been considerable evidence implicating the mitochondria in the apoptotic process; moreover, regulation of programmed cell death is considerably affected by the state of the mitochondria. However, other aspects of the mitochondrion *per se* have been noted to be altered in cancer, and may contribute to the neoplastic phenotype. Indeed, changes in mitochondrial DNA (mtDNA), as well as the dependence of cancer cell function on this organelle (despite the use of glycolytic pathways for energy production) suggest that the mitochondria are playing additional roles in the tumourigenic phenotype. This review describes findings of mitochondrial involvement in the tumourigenic phenotype, particularly in gliomas, and correlates the evidence to findings in other cancer types. The

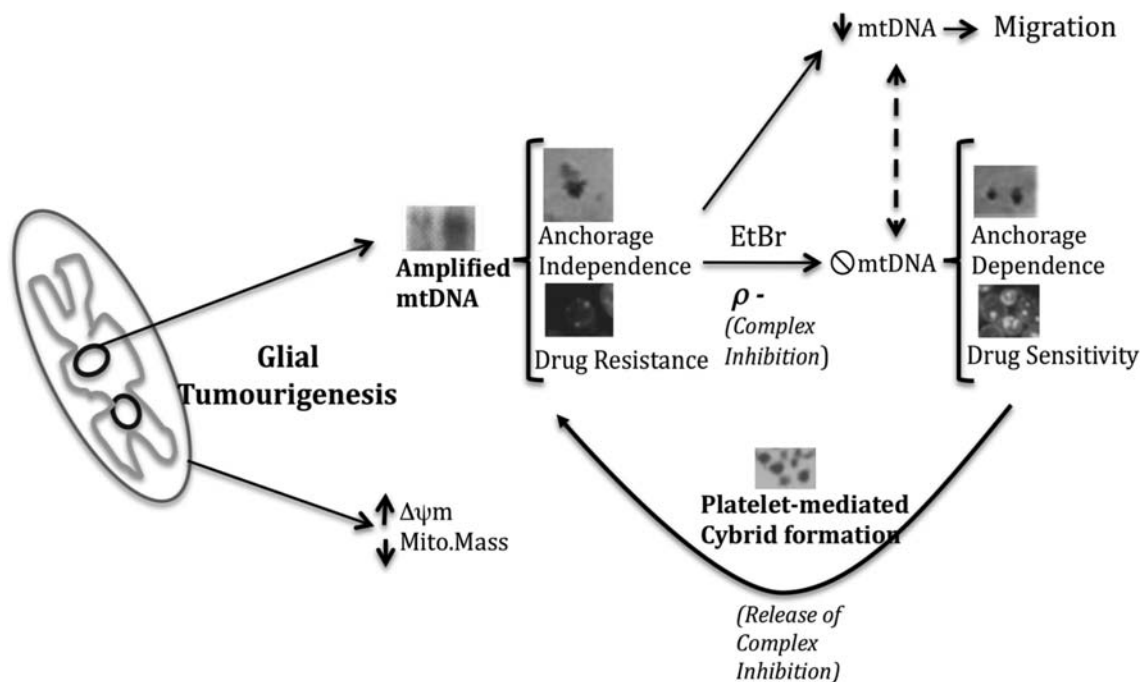


Figure 1. Model of involvement of the mitochondria in the tumourigenic phenotype. Gliomas show evidence of ontologically-early mitochondrial DNA (mtDNA) amplification, associated with mtDNA promiscuity into the nucleus, as well as an increased mitochondrial membrane potential ($\Delta\psi_m$) and decreased mitochondrial mass. With significant reductions in mtDNA levels by the formation of ρ^- cells [via treatment with ethidium bromide (EtBr) or complex inhibition], cells lose the ability to grow in an anchorage-independent manner, and become more sensitive to the apoptosis-inducing effects of cytotoxic agents. Less significant reduction (potentially due to the presence of other nuclear or environmental influences) shows a potential for the development of a migratory phenotype. Creating cybrid cells with normal platelets (and thus, normal mitochondria and mtDNA) result in a return of the *de novo* tumourigenic properties, and amplified mtDNA. Similar findings have also been noted in epithelial carcinogenesis.

evidence of mitochondrial involvement in the tumourigenic phenotype in gliomas is summarised in Fig. 1.

2. Alterations of mitochondrial DNA

mtDNA amplification in gliomas. Cancer arises from the accumulation of DNA and cytoplasmic abnormalities, which allows for the expression of the tumourigenic phenotype, which is manifested by certain elements, such as lack of terminal differentiation, immortality, anchorage-independent growth and resistance to cellular death from cytotoxic and further inducing stimuli (3). Gene amplification (of nuclear genes) is an important aspect of this multistep pathway to the neoplastic phenotype, and occurs in most, if not all, tumours (4). Indeed, cytogenetic and molecular cytogenetic data suggest this genetic finding is an intermediate step in the progression of cells in the multistep pathway noted above. Using a molecular subtractive hybridisation approach (5), an amplified cDNA was isolated which revealed a mitochondrial (cf. nuclear) sequence from a glioblastoma cell line. Whilst mtDNA had been observed to be structurally abnormal in the past (6,7), no studies had noted significant changes in copy number. Using mtDNA probes, different stages of primary tissues of glioma (low grade, pilocytic tumours, astrocytomas; intermediate grade, anaplastic astrocytomas; high grade, glioblastomas) as well as tissue culture lines of high grade gliomas, were evaluated to determine the relative ontogeny of the findings (8). Genomic DNA of these tissues revealed frequent amplification of not only the cell lines, but also the primary tumours; of significance was the finding that lower

grade tumours also harboured the amplification of mtDNA, suggesting this was an early event in the multistep pathway towards malignancy, in distinct contrast to nuclear DNA amplification in cancer, which is generally considered to be an intermediate step. This correlated with the expression data in a subset of tumours in which RNA was available; the data revealed an increased level of expression of the cDNA in both lower grade and cell line samples.

In gliomas, not only amplification, but also deletions, have been frequently noted. Components of the electron transport chain (including ND1 and ND2, *viz.* amino acid positions 4121-5275) have been reported to be deleted, correlating to diminished expression (8). Indeed, Dmitrenko *et al* (9) noted reduction of mitochondrial gene transcription in glioblastomas compared to that of the normal brain, using a differential cDNA screen. Similarly, they found ND1 to be consistently diminished in expression; further, ATP6, COXII, COXIII and 12S rRNA showed a decreased expression level. Kirches *et al* (10) found variations in the polycytosine tract; indeed, base substitutions were homoplasmic in nature. Similar results were noted by others (11), with the additional finding that there was no association between mtDNA and nuclear instabilities, an observation suggesting that in gliomas there are different distinct DNA repair mechanisms. Nevertheless, both studies suggest that the tumour-associated changes in mtDNA polymorphisms are via the same or similar mechanisms which generate such polymorphisms in an inherited manner. The pathogenic role of such alterations is yet to be determined, and requires additional correlative molecular and clinical studies (12).

These findings, *viz.* alterations in the sequences of the regulatory and coding regions of mtDNA in gliomas and the ontologically-early identification of amplification and heterogeneous areas of mtDNA amplification (suggesting deletions in the mitochondrial genome), have served to expand and extend early findings of abnormal mtDNA structure in cancer (13,14). The authors speculated on a possible association with petite mutants in yeast (15,16), where truncated mtDNA is associated with a dysfunction of the mitochondrion and with high degrees of amplification of the remaining DNA (see below).

In support of these studies in glioma, Penta *et al* (17) noted that breast, liver and colon cancers showed increases in mtDNA expression, with deletions in other tumours including those of the colon, stomach, bladder, head, neck and lung. Carew and Huang (18) also identified mtDNA as being altered in these tumour types, and additionally described findings in ovarian, esophageal, pancreatic, renal cell and prostate cancer, as well as thyroid cancers and haematologic malignancies. Beheshti *et al* (19) found mtDNA amplification in neuroblastoma, and Haugen *et al* (20) found an increased expression of mtDNA-encoded genes in papillary thyroid carcinomas. Jia *et al* (21) showed increases in mtDNA levels with the *mdr-1* genotype, and Tarantul *et al* (22) found evidence for amplified mitochondrial sequences in SIV-infected primate non-Hodgkin lymphoma (NHL). Similarly, Nikolaev *et al* (23) showed mtDNA increases in simian NHL, and Nenasheva *et al* (24) found that such sequences increased in SV40-transformed fibroblasts, observations suggesting that such modifications serve as early markers for the development and/or the pathogenesis of malignancy. However, Dani *et al* (25) evaluated breast, colorectal, gastric and head and neck tumours for the mtDNA⁴⁹⁷⁷ deletion ('common deletion'), and concluded that cells of these tumour types are essentially free of this mutation; similarly, Kiebish and Seyfried (26) did not find significant mtDNA mutations in mouse brain tumours and concluded that these mutations do not play a role in either chemically-induced or spontaneous mouse brain tumours. However, this has not been confirmed by others; indeed, primary cells of these types showed alterations in the mtDNA⁴⁹⁷⁷ region, amongst other mutations (10,27-31). Furthermore, the D-loop region (where mtDNA initiates replication, and is non-coding) has frequently been associated with alterations in the copy number as well as with mutations in osteosarcoma, uterine serous and gastric carcinomas, and ovarian, endometrial and breast cancers (32-39). Indeed, Duncan *et al* (40) identified a novel human mitochondrial D-loop RNA species which was up-regulated subsequent to cellular immortalisation, supporting the hypothesis that mtDNA alterations represent an early modification in generation of the tumourigenic phenotype. Moreover, associations of D-loop mutations have been noted in the background of the *p53* mutation, which is associated with changes in the expression of mitochondrial proteins (41,42). Of note, others have found a distribution of mutations throughout the mitochondrial genome (43,44). These studies support a role for mtDNA in the tumourigenic phenotype, and suggest alterations in the copy number (particularly increases) as an ontologically-early aspect of the pathway to malignant behaviour.

Promiscuous mtDNA. The discovery of mtDNA amplification, and the hypothesis that this form of pathobiology could recapitulate findings in yeast, suggested the possibility of 'mtDNA escape' in cancer. In yeast, mtDNA escape occurs as a manifestation of communication between the nuclear and mitochondrial genome (45,46), and has been postulated to be related to both genetic and environmental control. mtDNA has been found transposed to the nucleus in cultured cells (HeLa), as well as carcinogen-induced hepatic carcinoma cells (47,48). Indeed, it has been hypothesised that high copy numbers of mtDNA may be required to transfer mitochondrial sequences from mitochondrial sources to the nucleus in cancer, a phenomenon similar to that observed in yeast (49,50).

Using interphase nuclei with fluorescent DNA probes (interphase fluorescent *in situ* hybridisation, or 'interphase FISH'), Liang and Hays (8) evaluated both primary and tissue culture specimens of glial tumours, including lower grade tumours. As noted previously, mtDNA showed increases in copy number in all the lower grade tumours studied, but not the normal brain specimens. When evaluated by the interphase FISH technique using fluorescent probes of the entire mitochondrial genome, at least 69% of the nuclei revealed mitochondrial sequence hybridisation in the specimens investigated. In comparison, when evaluating two normal brain samples, as well as a glioblastoma specimen (which did not reveal increases in mtDNA copy number), <8% of nuclei revealed hybridisation with the mtDNA fluorescent probes. This observation suggested an association with the amplification of mtDNA, and the finding of 'promiscuous' mtDNA sequences in the nuclear compartment. Indeed, this was the first description of an association of the nuclear localisation of mtDNA with mtDNA amplification as an ontologically early event; furthermore, it was the first description of such an event found in primary tissues, rather than in cell culture experiments. Of particular interest is the recent finding by Guescini *et al* (51) who described microvesicles released by glioblastoma cells (as well as astrocytes), which harbour mtDNA. Such microvesicles carried Tsg101, CD9 and Alix markers, suggesting these are true exosomes; hypothetically, mtDNA can be transferred not only within, but also between cells, a finding which serves to provide intriguing potential mechanism(s) of disease processes, such as cancer.

Additional evidence of a role of promiscuous mtDNA in cancer has been reported. Czarnecka *et al* (52) have described cancer as a 'mitochondriopathy', and have noted alterations of mtDNA in various tumours, as well as the insertion of mtDNA sequences into the nuclear genome, with findings in hepatoma and meningioma (53-55). Interestingly, in both human myeloid leukemia HL60 and colorectal carcinoma COLO 320DM cells, amplification of mtDNA was found to be associated with mitochondrial sequences identified in the nucleus; in normal fibroblasts and peripheral blood T-cells, no promiscuous mtDNA sequence was noted (56). Such mtDNA sequences were found to be localised to the double-minute sequences identified by molecular cytogenetic techniques. Similarly, in mouse and rat model systems, mtDNA-like inserts were found at higher frequencies in tissue nuclei when expressed relative to those of normal cells (57). Additionally, Ricchetti *et al* (58) found mtDNA sequences

interrupting intronic regions of MADH2, a suppressor gene in colorectal cancer (59), and Borensztajn *et al* (60) and Turner *et al* (61) showed promiscuous mtDNA in the nuclear genome in both the intron and exon regions, which were associated with clinical phenotypes of disease (Factor VII deficiency and Pallister-Hall syndrome, respectively). These studies suggest that promiscuous mtDNA may represent a mechanism related to the generation of the tumourigenic phenotype. These mtDNA alterations could have significant implications regarding both the developmental mechanisms and detection of malignant cells in patients with cancer. Indeed, Copeland (62), Modica-Napolitano and Singh (63), Saffroy *et al* (64), Chatterjee *et al* (65), and Paul and Mukhopadhyay (66), have noted the relevance of mtDNA mutations and the potential consequences regarding both the diagnosis and treatment of patients with malignancy. In fact, the diversity of changes throughout the mitochondrial genome (67,68), in addition to specific polymorphisms (69,70), have been reported to be associated with the risk for cancer. Jiang *et al* (71) found that there was an increased mtDNA content in saliva associated with head and neck cancer patients. Treatment of these patients with apoptosis-inducing therapy (but not surgery alone) was associated with a decline in mtDNA levels (72). Hence, alterations in mtDNA may serve as a diagnostic tool, reflecting the response to a therapeutic intervention with modalities such as radiation therapy. Indeed, Polyak *et al* have filed a patent to utilise the presence or absence of mtDNA mutations in order to detect cells as a diagnostic approach in cancer patients (U.S. Patent Application 10/053,611).

3. Models of tumour phenotype

Creation of mtDNA-less cells (q^-). Cavalli *et al* (73) employed techniques to decrease the levels of mtDNA (q^- cells). In this manner, the direct effect of mtDNA, and mitochondrially-encoded biomolecules, would be evaluable, and the concepts could potentially be translated to the treatment of malignant disease.

Using a glioblastoma cell line, neoplastic cells were treated with low concentrations of ethidium bromide (EtBr). As EtBr is an inhibitor of mtDNA replication (74), during cell growth and passage, the initial number of mtDNA molecules was decreased by a factor of two upon each doubling. The cells became auxotrophic on uridine and pyruvate, a key characteristic of mtDNA-less cells. The requirement for uridine is related to the need of dihydroorotate dehydrogenase (an enzyme of pyrimidine biosynthesis, located on the inner mitochondrial membrane) to have an active electron transport chain for normal function. The auxotrophic dependence of q^- cells on pyruvate is not definitively known or well described, but most likely relates to the inability of the cell to oxidise cytoplasmically-produced NADH.

Cybrids (cytoplasmic hybrids) of the q^- cells were also created. Cybrids, fusions of q^- cells and normal cells, harbour mitochondria in order to replace this organelle and allow the study of mitochondrial function. In this case, normal platelets were employed, rather than *de novo* enucleated glioma cells and cytoplasm transformation, in order to avoid nuclear and cytoplasmic component influences of the tumour cells on the cybrid phenotype, i.e. entirely normal mitochondria with a

minimal level of influence from nuclear-encoded genes. By definition, platelets are fragmented aspects of cells, and thus anuclear.

A key aspect of the tumourigenic phenotype is the ability of such cells to grow in an anchorage-independent fashion (75). Both q^+ parent and q^- cells were assessed with regard to their abilities to form colonies in soft agar, an indication of anchorage-independence. Whilst both types of cells were able to attach to plastic tissue culture flasks, the formation of colonies occurred only in the q^+ parent cells that harboured mtDNA, with minimal colony formation found in q^- cells; similar results were noted in xenograft experiments.

Moreover, studies involving sensitivity to cytotoxic agents showed interesting results. Glioblastoma cells were treated with 1,3-bis-dichloroethyl-nitrosourea (BCNU), a common drug utilised in the therapy of patients with glioblastoma. q^- cells were more sensitive to the respective agent than parent q^+ cells. No changes were noted in the expression of *bcl-2*, *bax*, *mdr1*, *mrp* and *O⁶-alkyltransferase*. Interestingly, removal of functional mitochondria via depletion of mtDNA failed to prevent apoptotic cell death, an observation indicating that actively-translated, mitochondrially-encoded molecules are not, *per se*, required for apoptosis induction by cytotoxic chemotherapeutic agents.

Cybrids created from the q^- cells and normal platelet mitochondria revealed amplified mtDNA levels similar to q^+ cancer cells, suggesting that a cellular influence to maintain increased mtDNA levels was still acting through the q^- state, and was subsequent to platelet fusion. Glioma cybrid cells showed a return of anchorage-independent growth; as well, the relative sensitivity of cells to the effects of cytotoxic drugs increased to the levels of the parental cells.

Mitochondrial proteins have recently been found to be relevant in glioma. Indeed, the isocitrate dehydrogenase genes, IDH1 and IDH2 have been found to be mutated in astrocytomas (76); whilst IDH1 is a cytoplasmic protein, IDH2 resides in the mitochondria. Moreover, further studies (77) have shown IDH2 mutations to be more frequent in astrocytomas with oligodendroglial traits. These data are of interest in defining mitochondrial proteins and processes important in glial tumourigenesis; indeed, Hao *et al* (78) also reported mitochondrial protein alterations associated with tumourigenicity in paraganglioma. This group described alterations in the succinate dehydrogenase (SDH) complex, which is involved both in the electron transport chain and the tricarboxylic acid cycle (TCA); in particular, SDH5 was associated with a loss of SDH-dependent respiration, and a germline loss of function in a family with hereditary paraganglioma, an observation strongly suggesting SDH5 as a tumour susceptibility gene. The other components of this complex have also been found to be associated with this tumour type (79).

The importance of the mitochondria on tumourigenicity has been reported in additional studies. Hayashi *et al* (80) used enucleation studies and revealed that replacement of mitochondria from normal cells (a rat embryonic cell line) supported the growth of such rat glioma cybrids in nude mice. Indeed, growth patterns were identical in xenografts of the *de novo* rat glioma cells and in those of the cybrid subclones, although there was a heterogeneity of mitochondria

from both tumour cells and normal cells. Interestingly, Griguer *et al* (81) showed CD133 (a neural stem cell marker) up-regulation in ϱ^- cells; using similar cybrid techniques, it was observed during hypoxic conditions that the reduction of mtDNA levels was associated with a migratory phenotype; in such conditions, nestin (another stem cell marker) was also expressed. Of particular note was the ability to detect mtDNA by PCR, suggesting the cells may not have been entirely ϱ^- , accounting for the migratory phenotype (see below). The use of enucleated glioma cells to create cybrids from the ϱ^- cells resulted in the return of the CD133 levels of the parent cells. These data suggest that the milieu of glioma cells may be of significant influence, and the mitochondria may respond to generate a differential phenotype (e.g. migration) or cell surface expression (e.g. CD133). Such phenomena have also been noted in other tumour types (see below).

Earlier studies have shown the importance of mitochondria on tumorigenicity, as manifested by xenograft growth (82-84). Xue *et al* (85), using Boc-Aspartyl(O-methyl)CH₂F, removed mitochondria from neurons and HeLa leukemia cells; such removal did not damage other cellular components, but irreversibly committed these cells to death via an apoptotic mechanism. Hence, cells with inhibited or removed mitochondria, undergo apoptosis more readily compared to control cells. Dong *et al* (86) showed that mitochondrial transcription factor A (mTfam), a key regulator of DNA transcription, was significantly overexpressed (over 10-fold when compared to normal liver) in Morris (mouse) hepatoma cells, with augmentation of downstream mitochondrial gene expression. Moreover, mTfam protein was found in the hepatoma cell nucleus, suggesting a role of nuclear-mitochondrial communication in this neoplastic lesion. Indeed, these findings indicated that the mitochondrial influence on the observed tumorigenic phenotype is complex and involves a myriad of differing proteins interacting in both upstream and downstream pathways. Grandemange *et al* (87) performed a study, in which human dermal fibroblasts acquired transformed properties (morphologic changes, anchorage-independent growth, growth in xenografts) with stimulation of mitochondrial activity, using p43. Moreover, this transformation was associated with an increase in *c-Jun* and *c-Fos* expression, and virtual extinction of the tumour suppressor genes, *p53*, *p21^{WAF1}* and *Rb*. When mitochondria were then inhibited using chloramphenicol, a loss of the tumorigenic phenotype was observed (although restoration of the tumour suppressor genes did not occur). This study thus identified p43, at least in dermal fibroblasts, as a potential mechanism by which the early involvement of mitochondria in the tumorigenic phenotype may be manifested, and supports the hypothesis of a direct involvement of the mitochondria in maintenance of the tumorigenic phenotype. Similarly, additional studies in other model systems have identified proteins which may be responsible for the importance of the mitochondria in the malignant phenotype. In a clinically-based study, Ambrosone *et al* (88) found an association of manganese superoxide dismutase (MnSOD, a protein transported to the mitochondria) genotypes (with limitations on transport into mitochondria) with breast cancer risk; Roy *et al* (89) noted estrogen-related processes in the nuclear-mitochondrial communication, via estrogen-induced mitochondrial oxidants; Palomares *et al*

(90) showed a reduction of glutathione (GSH)-sensitised melanoma cells to cyclophosphamide; Ohnami *et al* (91) demonstrated that *K-ras* impacts the expression of mitochondrial genes; when utilising an antisense approach to pancreatic cancer cells, they identified 11 overexpressed genes, which were all of mitochondrial origin. Indeed, Weinberg *et al* (92) showed the requirement of mitochondria for *K-ras*-mediated tumorigenicity and in addition, the importance of mTfam in the phenotype. This further suggests the importance of the nuclear-mitochondrial communication pathway, where K-ras represents an important mediator and responder of mitochondrial gene expression and of changes in phenotype. Mills *et al* (93) identified CD14 as a potential mediator of apoptosis in HL60 cells. Mitochondria were inhibited using rotenone, antimycin A or oligomycin; all agents increased the expression of CD14 in these leukemic cells, followed by a loss of viability and apoptosis. Magda *et al* (94) studied A549 ϱ^- cells (lung cancer) induced by exposure to EtBr (although auxotrophy was not reported in the study). This group showed a lower proliferative ability, but a greater level of intermediate filaments expression in the ϱ^- cells, suggesting a more invasive phenotype. This observation is interesting in light of the hypothesis by Felty and Roy (95) that malignant cell spreading may be, at least in part, ascribable to the contraction and expansion of mitochondria, rather than only to the tension maintenance on the cytoskeleton via binding to the extracellular matrix. Moreover, these A549 ϱ^- cells, whilst able to grow in mouse xenografts, required longer induction periods with smaller tumours than their ϱ^+ parents. This suggests a clear difference in phenotype, perhaps due to differences in nuclear gene expression (96-98). Similar results have been noted in breast cancer cells (99). Interestingly, ϱ^- cells increased the expression of MHC-1 in addition to that of glucuronidation genes, a consideration relevant in immune surveillance and detection function; this suggests the relevance of mitochondria to the extrinsic manifestations of apoptosis induction, an observation similar to that made by Mills *et al* (93) with the expression of CD14, and to observations with CD95 (Fas) (100).

Gradual reductions of mtDNA levels in rhabdomyoblasts (transformed muscle cells) using EtBr resulted in changes in mitochondrial membrane potential ($\Delta\psi_m$; see also below) and in elevated free Ca²⁺ concentrations in the cytosol (101,102); these changes resulted in the alteration of genes related to Ca²⁺ transport and storage, which could be reverted once cells were allowed to grow in the absence of EtBr. Interestingly, as mtDNA levels were declining (but not absent), there was an increase in the ability of the rhabdomyoblasts to move through a reconstituted Matrigel membrane, an observation suggesting a migratory phenotype which was lost once mtDNA levels were allowed to return to their control values [see also Griguer *et al* (81)]. These data suggest mtDNA levels are affected not only early in the tumorigenic phenotype, but also in ontologically-subsequent manifestations of malignancy, and could serve as a marker identifying a pathway by which the migratory phenotype is induced. Hence, using feedback to the mitochondria and mtDNA levels, the nuclear-mitochondrial communication could effect the expression of nuclear genes in response to the micro-

environment milieu; this occurs in yeast at a variety of q^- settings (103,104).

Ethyl-nitrosourea (ENU)-transformed normal human astrocytes. Few studies have been performed evaluating the ontogeny of mitochondrial alterations in gliomas. A model of chemical transformation with normal human astrocytes (NHA) treated with ENU to derive transformed astrocytes was employed to evaluate the ontogeny of malignant changes (105,106). This model system was also tested with citrate, an allosteric inhibitor of phosphofructokinase (PFK), which represents a key regulatory enzyme in the glycolytic process. NHA exposed to ENU became immortal, and at approximately 26 doublings achieved the ability to grow in soft agar and at a low density. When NHA cells were treated with ENU and both pre- and co-treated with the PFK inhibitor, citrate, cells still became immortal, dividing well-past terminal differentiation. However, despite achieving the ability to avoid replicative senescence, these dually-exposed cells did not gain anchorage independence nor the ability to grow at low density. When ENU-transformed clones were subsequently treated with citrate (cf. pre- and co-treatment) and evaluated for growth under low density conditions, there was a decrease in colony formation observed at an added level of 100 μ M of this agent, suggesting that in this model, alterations of the malignant phenotype could be accomplished via an inhibition of PFK.

In NHA cells, $\Delta\psi_m$ was found to progressively decrease with increasing doublings until terminal division. Subsequent to undergoing 3-5 doublings, the ENU-exposed cells exhibited a significantly higher polarized $\Delta\psi_m$, when compared to that of NHA cells. Although the $\Delta\psi_m$ levels of these ENU-exposed cells declined, they were, in general, higher than those observed with NHA, with maintenance of an elevated membrane potential after the development of immortality. Interestingly, in parallel with the lack of anchorage-independence and growth at low density, pre- and co-exposure of cells to citrate did not result in the increased $\Delta\psi_m$ observed in cells treated with ENU alone. No differences were noted between the ENU/citrate-exposed cells, and the NHA or NHA citrate-exposed cells in $\Delta\psi_m$.

With respect to mitochondrial mass, NHA cells showed a very gradual, modest decline with successive doublings. With ENU administration, however, after 10 doublings, there was a substantial reduction in mitochondrial mass, to about 25% of the starting mass measured. Citrate prevented this dramatic drop in mitochondrial mass; even with immortality achieved, the mitochondrial mass of ENU/citrate-treated cells did not differ from NHA cells which were terminally differentiated. When calculating the $\Delta\psi_m$ /mitochondrial mass ratios, such data revealed a hyperpolarized state of the mitochondrial membrane of the ENU-transformed cells, which was preventable by citrate pre- or co-exposure. These data suggest that hyperpolarization of the mitochondrial membrane represents an early manifestation of the transformed/tumourigenic phenotype, associated with anchorage independence, and may be mitigated by the use of the PFK glycolytic inhibitor, citrate.

Hence, maintenance of the tumourigenic phenotype was found to be related to early changes in $\Delta\psi_m$ in a glioma model.

Noteworthy, is the observation that in neu-initiated mammary epithelial cell tumours with increased $\Delta\psi_m$ values, the inhibition of glycolytic pathways using short hairpin RNAs of LDH-A, decreased $\Delta\psi_m$ and also reduced the ability of these cells to proliferate in both *de novo* and hypoxic environments (107), supporting glioma data acquired with the use of citrate as a glycolytic inhibitor of PFK, with associated changes in membrane potential reflective of the overall tumourigenic phenotype (i.e. resistance to apoptosis and growth in xenografts). Other researchers have investigated similar hypotheses (108). Matarrese *et al* (109) using a drug-resistant human T-lymphoblastoid CEM cell line, VBL100 variant (with amplified mtDNA), sensitive to TNF- α , showed that the induction of apoptosis was attributable to the depolarization of $\Delta\psi_m$ *per se*. Indeed, these authors showed a direct correlation between susceptibility to apoptosis (by various agents) and the state of the mitochondria ($\Delta\psi_m$); prevention of the decrease in the membrane potential, and thus of mitochondrial function, using bongokrekic acid (an inhibitor of the $\Delta\psi_m$ decrease, acting by antagonising the adenine-nucleoside transporter, which, in turn, is responsible for allowing the mitochondrial-membrane permeability transition and thus an attenuation of the potential), showed that the apoptosis-inducing effects could be mitigated by agents which affected the intrinsic (i.e. the mitochondrial), but not the extrinsic pathway of apoptosis.

4. Targeting the mitochondria as a translational therapeutic approach

Previous studies have noted that the involvement of the mitochondria and the susceptibility to apoptosis are separated from ATP production via oxidative phosphorylation, which was diminished (110). Further chemotherapeutic approaches with agents which were noted to have anti-mitochondrial activity have been described. 2',3'-Dideoxycytidine (ddC) an anti-retroviral agent, is a potent inhibitor of DNA polymerase γ and consequently has a distinct anti-mitochondrial activity (111). ddC is currently approved for patients with acquired immune deficiency syndrome (AIDS) as part of the multi-agent cocktail treatment regimen. ddC was evaluated in glioma cell lines (112). Alterations of $\Delta\psi_m$ were noted, with a decrease in polarized mitochondria from 31-84% in cells treated with ddC; at all ddC doses studied, cell death eventually ensued. Interestingly, at some doses of ddC (between 10 nM and 15 μ M), a delay (but not total rescue) in the death of both cell lines could be obtained with supplementation of the medium with glucose, uridine and pyruvate, an observation suggesting that the cells lacked a fully functional respiratory chain; however, cells treated with higher doses of ddC were unresponsive to such supplementation. This suggests that mitochondria (in a functional q^- state) are involved in at least one mechanism of the observed ddC-induced cellular death, although since rescue was only partially possible with glucose, uridine and pyruvate, and the cells eventually died, the overall mechanisms effecting apoptosis are likely to be more complex and multifactorial.

To more specifically target the mitochondria, compounds which have lipophilic (i.e. diffusible into the cell and past mitochondrial membranes), and cationic (allowing attraction and retention in the anionic environment of the mitochondrion)

characteristics would be ideal. A compound which fits these essential criteria includes tyrphostin AG17 [NSC 242557, (3,5-di-tert-butyl-4-hydroxybenzylidene) malononitrile], described by Burger *et al* (113) and available from the Cancer Therapeutics Evaluation Program (CTEP), National Cancer Institute, NIH, USA. This agent also has tyrosine kinase inhibitory activity, which is manifested as a growth retardation mechanism in other studies (114); it has been studied in human glioma cell lines as a potential sensitiser to the effects of cytotoxic chemotherapy (115). Treatment of glioma cell lines demonstrated auxotrophy on uridine and pyruvate after 30 days of exposure, which was not observed with NHA. Exposure to AG17 after 2 or 3 days was associated with an increased sensitivity to the cytotoxic agent BCNU; in contrast, the sensitivity of NHA cells to BCNU was not modified (LD₅₀ of 40 µg/ml for both the AG17-treated and the untreated cells).

In other pharmacologic approaches, Lena *et al* (116) evaluated temozolomide-resistant glioma cells, using agents to induce the mitochondrial permeability transition (MPT). Selected disrupting agents used included betulinic acid, lonidamine and CD437; each was able to dissipate $\Delta\psi_m$ within 24 h of treatment. Such dissipation was inhibited by the MPT pore blocker, cyclosporine A. Subsequent evaluation of treated glioma cells revealed a mixture of cell death mechanisms, although the vast majority of cells underwent apoptotic cell death. These experiments suggested that the alteration of the MPT in temozolomide-resistant glioma cells effected an apoptotic phenotype; since evaluated cells included a PTEN hemizygous deletion and a lack of EGFR amplification, this approach might be useful when targeted therapy is otherwise unavailable. Griguer *et al* (117) studied OMX-2, a methyquinone targeting mitochondria; OMX-2 was able to dissipate $\Delta\psi_m$ without effecting cell death in a glioma cell line; in contrast, normal cells maintained their membrane potential despite exposure to the agent. Moreover, OMX-2 was able to also potentiate cell death induced by 2-deoxyglucose in glioma cells. Higgins and Pilkington (118) were able to demonstrate an increased sensitivity of glioma cells to the inhibitory effects of dexamethasone and of tricyclic drugs, particularly clomipramine, on respiration. The authors suggest the use of such agents for targeting the mitochondria could be useful in the development of therapies for gliomas. Similarly, the use of glitazones (119) and carvedilol (120) revealed a potential to create apoptosis-sensitisation strategies with other agents (both via targeted and cytotoxic drugs) against glial tumours. Finally, sensitisation of glioma cells using interferon γ was related to the engagement of Fas, when up-regulation of mRNA expression was noted. Given data on α cells expressing increased levels of Fas, it would be interesting to evaluate the relationship of the cytokine to mitochondrial function (100). Regardless, it would be required to take into account the sensitivity to Fas-based apoptotic mechanisms, given the lack of responsiveness to Fas-engagement in some tumour types (121,122).

Lund *et al* (123) were able to demonstrate the direct effects of ddC on mtDNA copy number. Indeed, there was a significant depletion of mtDNA with ddC treatment in HepG2 hepatocellular carcinoma cells, without this agent exerting an effect on the metabolism of such cells. These results confirm

data acquired in glioma cells, which became auxotrophically-dependent on uridine and pyruvate when treated with ddC. Similarly, Bouayadi *et al* (111) showed that ddC could effect apoptosis in B16 melanoma cells, and related this finding not only to DNA polymerase γ inhibition (found in mitochondria), but also to DNA polymerase β (which is typically found to be down-regulated in normal cells, but overexpressed in some). This phenomenon was also observed by Louat *et al* (124), and suggests that ddC has a multitude of functions by which its anti-neoplastic activities are manifested. However, it is clear that a significant, and probably dominant, effect of this agent is through the mitochondrion, since the sensitising effects of ddC to the apoptotic phenotype require a decrease in mtDNA levels/transcripts to a critical threshold level (125). Indeed, the relative resistance to ddC has been explored in CEM T-lymphoblast cells (created by incubation at a low concentration of the anti-nucleoside agent) (126); ddC-resistant cells showed an amplification of mtDNA relative to the corresponding *de novo* cells, with significant increases in the expression of mTfam. These data suggest that mTfam may be a mitochondrially-based mechanism of drug resistance to compounds such as nucleoside inhibitors as a manifestation of the tumourigenic phenotype. Previous work has also shown the importance of mTfam in cancer cells (86). Further, it has been shown that mutations in mtDNA polymerase γ promotes tumourigenesis in breast cancer (127). Moreover, ddC has also been noted to have sensitising effects with ionizing radiation. Indeed, Coucke *et al* (128) showed a dose-dependent response of ddC treatment to sensitization with radiation. This is of particular interest since radiotherapy generates reactive oxygen species (ROS) *in vivo*, including the aggressively-reactive hydroxy radical (\cdot OH). Inhibition of the mitochondrial processes could potentially diminish this effect. Moreover, Humer *et al* (129) showed a synergistic effect of azidothymidine (which has a similar anti-mitochondrial effect to ddC), with CDDP in melanoma cells. However, these results are confounded by the possible formation of a complex of azidothymidine with the platinum(II) [Pt(II)] metal ion centre in CDDP. Indeed, in experiments focused on evaluations of the synergistic inhibitory effects of these agents on melanoma cell growth, the agents were co-administered in the same medium and Pt(II) readily formed such complexes with thymidine (130). A further complicating factor is that CDDP can also undergo ligand-substitution reactions with selected amino acids (either free or protein-incorporated) or other biomolecules present in the culture medium employed, particularly methionine and protein residues of this amino acid which have a side-chain thioether group sulphur donor atom, for which Pt(II) has a high affinity. Mitochondria must, therefore, harbour further complex mechanisms outside of changes in expression which alter the phenotype of therapeutic resistance as a manifestation of the tumourigenic phenotype.

Clinically, the use of ddC in combination chemotherapy has shown evidence of efficacy. In adult patients with T-cell lymphoma, Besson *et al* (131) treated patients with CHOP induction (cyclophosphamide, hydroxyurea, vincristine and prednisone); in a subset of patients, this was followed by consolidation with etoposide and an anti-nucleoside (typically ddC, but in some cases azidothymidine). Patients treated with

the consolidation regimen showed a significantly increased survival when compared to those with induction only with CHOP (17 vs. 3 months, $p=0.004$); no major toxicity was noted, although there were some peripheral neuropathy events. Although this study was small ($n=29$), it showed the potential benefit of addressing multiple targets, including the mitochondria, in neoplastic diseases. In addition, patients with AIDS-related lymphoma were also treated with low doses of methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone, in addition to ddC, in a single arm study (132). Complete response was noted in 56% of patients, including those with poor prognosis factors (AIDS diagnosis prior to lymphoma; $<200/\text{mm}^3$ CD4 lymphocytes). Interestingly, minimal peripheral neuropathy was noted, and improvements in AIDS-related markers were observed. These observations suggest that ddC may indeed be valuable in the treatment of certain cancers, as noted herein; the key will be to ensure that the therapeutic index, particularly with the anticipated longer time of therapy and when administered in combination with other agents, is wide enough to avoid toxicity.

Lipophilic cations and related compounds should be valuable for the treatment of tumours with an increased anionic $\Delta\psi_m$ (133). Indeed, in view of their ability to diffuse into and be retained within the mitochondrial space, this may serve as the mechanism involved in studies which have revealed cellular chemosensitisation to such agents. Targeting mitochondria to enhance the potential sensitivity of cells to cytotoxic agents is of particular interest with respect to the design of new chemotherapeutic and other approaches with available as well as newly-developed novel agents. Indeed, such increases in sensitivity of tumours to chemotherapy could allow the disease process to be more accessible to therapeutic intervention, with limited toxic effects to normal tissues.

With the findings of changes in the tumourigenic phenotype related to the mitochondria, a great deal of interest has been focused on targeting organelle-related functions for the treatment of human malignancy (134). Several studies (110,135) have shown an increased sensitivity to CDDP in g^- U937 and 143B cells; in both studies, the mitochondrial pathway was not found to be the major producer of energy (using mitochondrial inhibitors), and in the latter, increases in sensitivity to CDDP were related to significantly increased caspase 3 activation. Indeed, these 143B g^- cells were also found to have an increased release of cytochrome c into the cytoplasm with exposure to CDDP, an observation suggesting that caspase activation, in addition to increasing the magnitude of the response to CDDP, plays a role in g^- apoptotic sensitivity. Moreover, Cummings and Schnellmann (136) showed at least partial (50%) reliance of CDDP-induced apoptosis on caspase 3 activation in normal renal proximal tubular cells, without changes in ATP concentrations; further potential mechanisms included those speculated to involve either bax or BID proteins, but most likely are not ascribable to caspases 8 or 9, which were inhibited in these studies. Similarly, Schwerdt *et al* (137), showed an increased sensitivity to CDDP when normal collecting duct-derived cells were incubated with mitochondrial inhibitors; again, no apparent modifications in ATP levels were observed. Troyano *et al*

(138) showed that ATP levels were maintained in U937 lymphoma cells when mitochondria were inhibited with oligomycin, indicating these cells are not dependent on mitochondria for energy production. However, of much interest was the additional finding that depletion of GSH (a reactive oxygen species scavenger, transported into the mitochondria) increased the sensitivity of cells to CDDP-induced apoptosis when mitochondria were inhibited, an observation similar to those of Kharbanger *et al* (139) and of previous studies conducted with g^- U937 cells. Moreover, with increases in the duration of CDDP exposure, a necrotic phenotype was noted, along with a decline in $\Delta\psi_m$. This suggests that GSH may serve as a candidate for moderating sensitivity, as well as being involved in the mechanism of mitochondrially-mediated cell death, and that mitochondria, at the extreme, may also play a role in necrotic cell death. In contrast, Yang *et al* (140) found that in head and neck cancer cell lines which were enucleated, there was no difference in the sensitivity to CDDP when compared to parental cell harbouring nuclei; however, g^- cells were more resistant to the effects of CDDP, i.e., up to five times more resistant to the effects of the drug. Similarly, Lee *et al* (141) found that when evaluating TRAIL-induced apoptosis in SK-Hep1 hepatoma cells, g^- cells were more resistant than parental cells to the effects of this cytokine; a different model (HeLa cells treated with photodynamic therapy or adriamycin) also showed resistance to apoptosis in g^- cells (142). However, these data were in contrast to the observations by Mizutani *et al* (143), which revealed that cybrids derived from mitochondria were more resistant to CDDP than g^- cells, an observation which agrees with previously cited results, as well as with data from Mizumachi *et al* (144) who reported that anthracycline-resistant head and neck cancer cells had a significantly higher mtDNA content. Furthermore, in alternative model systems, photodynamic approaches have revealed increases in cell death with mtDNA depletion (145,146); indeed, in L1210 murine leukemia cells, there was a significant increase in the sensitivity to mitochondrially-related photodamage in g^- cells, a process giving rise to apoptotic death; when made resistant to ddC, CEM lymphoblast cells showed increases in their mtDNA content and expression (126). These differences may arise from the various cell sensitivities to apoptosis (based on grade and stage, and thus nuclear gene expression, e.g. *bax:bak* expression), the effects of CDDP directly on the mitochondria, and/or from alterations in mitochondria during the development of CDDP-resistance, and hence its susceptibility to the Pt(II) ion-based chemotherapy. Several research groups have also proposed this hypothesis (110,140,141,147,148). Nonetheless, sensitivity to apoptotic-inducing stimuli, such as CDDP, can be manifested by the state of the mitochondria, which has direct therapeutic implications with regard to both targeting and combination therapies. In this regard, compounds such as tyrphostin AG17 could be of interest, since this agent exerts little or no effect on normal cells (astrocytes); Wallace and Starkov (149) noted that the selective delivery of compounds to the mitochondria, as speculated with AG17, could be a key opportunity for therapeutic intervention. This compound is of interest as a tyrosine kinase and as a mitochondrial inhibitor in cancer (150), including gliomas.

5. Discussion

The investigations cited herein have investigated the role that mitochondria play in the tumourigenic phenotype, outside and above the facilitation of apoptosis, and the potential of these phenomena for use as a target in the treatment of glial and other cancers. In gliomas, as well as in other cancers, mitochondrial alterations are ontologically-early processes and manifest throughout the multistep pathway to malignancy, with both nuclear genetic and genomic alterations, as well as those at the level of the mitochondria and its genome. These changes evolve over the lifecycle of the malignant cell and its milieu. Indeed, low grade gliomas show evidence of mtDNA increases, which was also noted by Nenasheva *et al* (24) in other haematologic neoplasms. In contrast, varying results have been reported in hepatocellular, lung, colorectal and gastric cancers, where some, but not all, types showed an amplification of mtDNA (141,151). Of particular interest was the association of the higher levels of mtDNA with less somatic mutations at the D-loop, and that mtDNA reductions occurred (with increases in D-loop mutations) in later cancer stages (141,151). Combined with the data described in gliomas and other tumours, these findings support a model where mtDNA amplification represents an early aspect of the generation and support of the tumourigenic phenotype, potentially ascribable to oxidative stress and altered nuclear-mitochondrial communication (as in petite mutants in yeast), followed by additional manifestations of malignancy, where glycolytic pathways predominate and nuclear mutations support tumourigenicity. While this would not obviate the maintenance of high mtDNA levels, the external and genomic milieu would determine whether the cell is required to have amplification (particularly, for example, in the context of certain types of drug resistance) (21,86,126,138). It has recently been noted that promiscuous mtDNA anomalies are of particular relevance at regulatory sequences in gene-rich locations of the genome, within both introns and exons, including, for example, transcriptional modulators and expressed genes responsible for diverse functions such as thiamine transporters, serine proteases and the T-cell receptor β -chain (58, Liang and Novak, unpublished data). Hence, alterations in mtDNA copy number and expression, as well as promiscuous mtDNA, is potentially an early event in the tumourigenic phenotype, being noted not only in gliomas but other malignancies as well. Moreover, epigenetic findings with mtDNA depletion have been observed, with aberrant methylation at promoter regions of genes putatively important in cancer (e.g. endothelin B receptor, O^6 -methylguanine-DNA methyltransferase, and E-cadherin) (152). Hence, this area of cancer biology, as well as the mechanisms upstream and downstream of mtDNA amplification, continue to be evaluated to improve our understanding of the mechanisms involved as well as the ramifications of this process in human cancer cells.

Evidence from mouse epidermal growth factor-transformed cells suggests that the initial influence on mtDNA copy number may be attributable to the regulation of nuclear-encoded proteases (e.g. the mitochondrially-located LON protease); similarly, heregulin, which activates the *erb-b2* pathway, can also increase expression of mtDNA genes, such

as COXII (153). On the phenotypic level, in colorectal cancer, a lower mtDNA content was associated with a later clinical stage, which in turn was associated with a reduced five year disease-free survival (154). In radiation-induced preleukemic lesions, increases in cytochrome c oxidase activity were noted; with maturation of thymic lymphomas, there was a reduction of COXI/II/III gene expression, data suggesting that the mitochondrial changes occur early in this process (155). Furthermore, in cells which harbour HMGA1 overexpression (associated with advanced malignancy and increased metastatic potential), mtDNA levels (as well as mitochondrial mass) were found to be reduced, although, interestingly, and consistently with the findings cited, the susceptibility to apoptosis was increased (particularly to inhibitors of glycolysis) (156). Indeed, Wu *et al* (157) showed that in gastric cancers mtDNA was depleted in the most infiltrating class of tumours. Collectively, these data suggest that mtDNA alterations, in particular increases in copy number and/or expression, are early in the manifestation of the tumourigenic phenotype, both with respect to ontogeny and stage of disease. With an infiltrating or migratory phenotype, levels of mtDNA appear to become normalised in some circumstances, suggesting a distinct difference in genotype/phenotype. This has been observed in a variety of other studies evaluating the biology of the migratory vs. growth neoplastic phenotypes (158,159). It could thus, be postulated that reversion back to a growth phenotype would effect the specific alterations noted previously, including changes in mtDNA levels and resultant phenotype.

As a future approach for therapy, there have been a significant number of studies now evaluating the targeting of the tumourigenic phenotype via the mitochondria both as therapy (141,151,160-162) as well as in a preventative approach (163-165). Petit *et al* (166), as well as Liminga *et al* (167), postulated that the toxicity of certain anti-mitochondrial compounds might be utilised to treat and/or sensitise cancer cells to the effects of other agents. Joshi *et al* (168) also targeted mitochondria using a novel approach with fatty acid agents (most likely to include the apoptosis-inducing agent ceramide) to effect cell death, which was interestingly dependent on an intact respiratory chain; Fantin *et al* (169) showed, using an approach previously described with AG17, that compound F16 with a delocalised positive charge would accumulate in the anionic mitochondria, effecting cell death in an upstream-independent manner. Indeed, with the data noted with ddC, there is some level of proof-of-concept that this approach could have significant viability in the future, and there are ongoing efforts to use this postulated approach to modify the tumourigenic phenotype in patients with cancer (170,171). Certainly, targeting mitochondria at both the early stages subsequent to the development of immortality, as well as at the time of the cell growth phenotype, would appear to represent the most prudent opportunities for therapy. In addition, if cells which harbour a migratory phenotype have a hyperpolarized $\Delta\psi_m$ (169), appropriate use of anti-mitochondrial agents such as lipophilic cations (e.g., AG17 or F16) could have significant utility when administered alone, or (more likely) in combination treatment regimens.

In summary, the mitochondria play a significant role in the tumourigenic phenotype, in addition to the mediation of

the intrinsic pathway of apoptosis, and represent an evolving approach for the therapy of cancer. Gliomas have been shown to represent both this malignant set of behaviours as well as to respond to interventions targeting mitochondria, both *in vitro* and *in vivo*. The ongoing efforts to understand and exploit these biologic findings will hopefully result in the improvement of outcomes for patients who harbour neoplastic disease.

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