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Review Article

The potential for strategies using micronutrients and heterocyclic drugs to treat invasive gliomas

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Summary

Local invasion of neoplastic cells into the surrounding brain is perhaps the most important aspect of the biology of gliomas that precludes successful therapy. Despite significant advances in neuro-imaging, neurosurgery and radiotherapy, the median survival for patients with a malignant glioma is still less than one year. With the increasing knowledge of the biology of brain tumours, derived from cellular and molecular studies, new methods of treatment are being developed with some success. Approaches studied already include anti-invasive, pro-apoptotic and anti-angiogenesis strategies and clinical trials are imminent.

In this article we review two new approaches to the management of gliomas: *nutraceutical* intervention and *heterocyclic* drugs. The first approach uses a combination of naturally occurring agents, including citrus flavonoids, chokeberry extract, red grape seed extract, lycopene, selenium and red clover extract. These agents can either trigger apoptosis or affect the pathways underlying diffuse invasion. The second approach involves the use of a heterocyclic drug, clomipramine, which selectively triggers apoptosis in neoplastic cells but not in normal glia. The article refers to the results of recent studies performed in our laboratory which suggest that these new approaches can be translated into benefit to patients.

Keywords: Therapy; gliomas; micronutrients; clomipramine.

Introduction: a therapeutic problem to solve

The incidence and mortality from intrinsic brain tumours have each risen sharply over the past three decades [44]. In adults these tumours are mainly derived from glial cells or their progenitors and are either high-grade malignant neoplasms or lower grade tumours that frequently progress to a more malignant form. Intrinsic, or primary, brain tumours usually do not metastasise to distant organs, instead, they are characterised by local invasion of the normal brain [43]. This feature is a major factor in the failure of current treatments. The mechanisms that underlie this characteristic invasive pattern include complex, interacting processes between cell adhesion molecules, extracellular matrix (ECM) components, proteases (e.g. matrix metalloproteinases MMPs), cytoskeletal elements, gangliosides, growth factors and cytokines.

Radiotherapy is, the only treatment that consistently improves survival time in this group of tumours. Chemotherapy may improve survival slightly but is associated with significant toxicity [37, 4]. Furthermore, the delivery of drugs to the invading cells can be difficult. Cytotoxic drugs, while entering into the main tumour mass, frequently fail to reach the invading cell population because entry into the normal brain is prevented by the blood-brain barrier (BBB).

In seeking new treatments, it is necessary to combat diffuse local invasion and to develop cytotoxic agents able both to cross the BBB and to be effective against both dividing and migrating cells. Potential approaches include anti-invasion, anti-angiogenesis and pro-apoptotic strategies.

One new approach uses a combination of 6 naturally occurring *micronutrients*; citrus flavonoids, chokeberry extract, and red grape seed extract, lycopene, selenium and red clover extract. These agents are believed to cross the blood-brain-barrier and can either trigger apoptosis or affect the pathways underlying diffuse invasion. A second new approach involves the use of a *heterocyclic* drug, clomipramine, that crosses the blood-brain-barrier and is sequestered in brain for long periods. It

selectively triggers apoptosis in neoplastic cells leaving the normal glia unaffected. In this paper we review the results of recent research, in our own and other laboratories and propose that each approach may become useful in patients.

Micronutrients in glioma management

Micronutrients (vitamins, minerals, and other agents found in food, such as flavonoids) have potential therapeutic effects in cell culture and animal models of peripheral cancers. A micronutrient is referred to as a *nutraceutical* when it is used at a pharmacological dose in treatment of a disease. This nutraceutical approach is one strategy for an alternative/adjuvant therapy in the management of a malignan brain tumour.

Flavonoids

Flavonoids are water-soluble, low molecular weight, naturally occurring, polyphenolic compounds and are widely distributed in plants. Flavonoids are primarily recognised as the pigments responsible for the many shades of colour found in most fresh fruit, vegetables and herbs. They occur in high concentrations in the juice and peel of citrus fruits. They are also prevalent in grapes, broccoli, onions, apricots, wine, green tea, soy products, cherries and grains [17, 18]. It is estimated that the daily intake of flavonoids in the Western diet ranges from 200 mg/day to 1 gram, a quantity that could provide pharmacologically significant concentrations in the body [41, 19].

Over 6,000 different, naturally-occurring flavonoids have been described to date [16]. The basic structure (the flavane ring system) consists of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (C) in the middle (Fig. 1). They can be sub-divided into a number of group but the criteria for these are not universally agreed. A recent system of classification includes 6 subclasses of flavonoids: flavonols, flavans, flavones, flavanones, isoflavones and anthocyanidins [39].

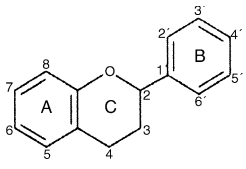

Fig. 1. Basic structure of flavonoids (flavane ring) consists of a C6 C3 unit (Ring B and carbons 2, 3 and 4) and a C6 unit (Ring A). Various subgroups of flavonoids are classified according to the substitution patterns of ring C

Although flavonoids were first isolated in the 1930s, their therapeutic potential has only been explored in the last two decades. The flavonoids have now been recognised to possess a range of potentially beneficial properties and been studied proposed to have anti-cancer, anti-inflammatory, antioxidant, antiviral and anti-proliferative activities [35, 19, 36].

Our original hypothesis was that naturally occurring citrus flavonoids (e.g. tangeretin from tangerines) could be potent anti-cancer agents in the treatment of brain tumours. Initial studies indicated such an efficacy could result from the ability of these agents to interfere with the mechanisms controlling invasion of the normal brain by tumour cells [46]. Subsequent studies assessed the potential value of synergistic effects of a combination of a number of micronutrients on malignant brain tumour cells *in vitro*. The combination included a citrus flavonoid (tangeretin), isoflavones from red clover (genistein and diadzein), a carotenoid found in tomatoes (lycopene), oligomeric proanthocyanidins from red grape seed extract, anthocyanins and catechins from chokeberry extract and the trace element selenium.

Tangeretin: citrus flavone

The anti-cancer properties of citrus flavonoids have been reviewed previously [8]. Different citrus flavonoids have different effects on the behaviour of tumour cells. Early studies of the citrus flavone, tangeretin, showed that it inhibited the invasion of MO4 cells (Kirsten murine sarcoma virus transformed fetal mouse cells) into embryonic chick heart fragments *in vitro*. These anti-invasive effects were reversed by omission of the molecule from the tissue culture medium [6, 5]. Subsequently, it was shown to be anti-proliferative in human lung carcinoma, human T-cell carcinoma, human gastric cancer [22], human squamous cell carcinoma, gliosarcoma [21], human breast cancer [15] and human fibrosarcoma HT-1080 cells [47].

Our own data suggest that tangeretin (Fig. 2) and nobiletin (which differs from tangeretin by only a

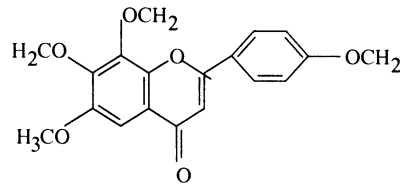

Fig. 2. Chemical structure of citrus flavone, tangeretin found in tangerine and orange-peel

methyl group), have potent anti-invasive effects *in vitro* on a variety of brain tumour cells [46]. Comparison of tangeretin with nobiletin, another citrus flavonoid, naringin and with the limonoid, limonin, showed that tangeretin had the most potent anti-invasive properties *in vitro*. Its effects may reflect down regulation of the proteases, particularly the matrix metalloproteases, known to mediate invasion. Recently, we have shown that tangeretin is able to cross the blood-brain-barrier in the rat brain. It was found in all of the major organs but was found to be concentrated in certain regions of the brain, so that concentrations in the hypothalamus being 6 fold higher than any peripheral organ [13]. These findings support tangeretin plans for clinical evaluation of micronutrients.

Red clover extract isoflavones: genistein and diadzein

Isoflavones are non-steroidal compounds with weak estrogenic activity. The compounds may compete with endogenous hormones and also may inhibit a number of enzymes involved in oestrogen metabolism [31]. Genistein (Fig. 3) and diadzein (Fig. 4) are the primary isoflavones found in red clover and legumes such as lentils, chickpeas and soyabeans. Soya is consumed in high amounts by Asian populations, (20–80 g per day), whereas the average western dietary intake is between 1–3 g. Interestingly, a number of epidemiological studies have shown a lower incidence of breast, colon and prostate cancers in Asian populations that eat a diet high in soya. [34]

Barnes *et al.* [3] have extensively reviewed the anticancer effects of genistein, using *in vitro* and *in vivo* models. In addition to genistein's estrogenic effects, it is also a potent and relatively specific inhibitor of the activity of epidermal growth factor receptor (EGFR) tyrosine kinase. This may be particularly relevant to the treatment of gliomas because amplification of the gene for EGF-R is seen in approximately 40% of glioblastoma multiforme (GBM) [28]. Few studies have looked specifically at the effects of isoflavones on brain tumours. Khoshyomn *et al.* [24] showed that genistein acted synergistically with cisplatin in its antiproliferative and cytotoxic effect in two medulloblastoma cell lines. Penar *et al.* [38] showed that genistein inhibited GBM infiltration mediated by EGF-R using *in vitro* cultures of human glioblastoma spheroids. Our pilot studies, using time-lapse video-microscopy suggest that genistein and diadzein isoflavones can reduce cell motility of brain tumour cells *in vitro* in a dose dependent manner (unpublished data).

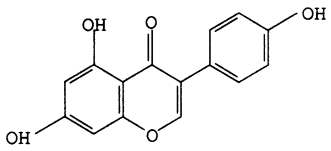

Fig. 3

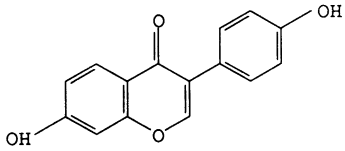

Fig. 4

Figs. 3 and 4. Chemical structures of the 2 main red clover extract isoflavones: genistein and diadzein, respectively

Chokeberry extract: anthocyanins and catechins

There are a number of flavonoids present in chokeberry extract which include the flavone rutin (quercetin-3-rutinoside) (Fig. 5), 3 anthocyanins; cyanidin-3-galactoside (Fig. 6), cyanidin-3-arabinose and cyanidin-3-xyloside, and 2 catechins ((+)-catechin (Fig. 7) and (−)-epicatechin (Fig. 8).

There are no reports that chokeberry extract has anticancer properties. Nevertheless, our preliminary studies show, that chokeberry is more effective in downregulating matrix metalloproteinase expression than bilberry or

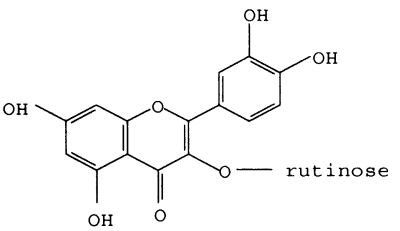

Fig. 5

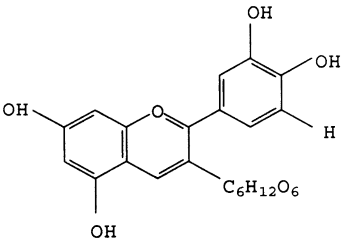

Fig. 6

Figs. 5 and 6. Chemical structures of the flavonoids found in chokeberry extract: flavone rutin (quercetin-3-rutinoside) and anthocyanins; cyanidin-3-galactoside, respectively

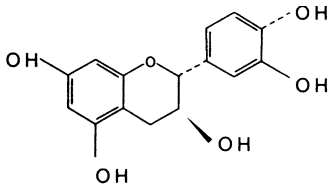

Fig. 7

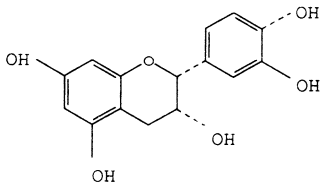

Fig. 8

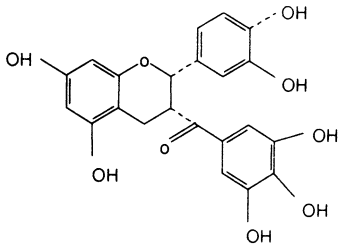

Fig. 9

Figs. 7–9. Chemical structures of the oligomeric proanthocyandins (made up of 3 monomeric sub-units) found in red grape seed extract: catechin (Fig. 7), epicatechin (Fig. 8), and epicatechin (-3-0-) gallate (Fig. 9). The former 2 monomers found in red grape seed extract are also present in chokeberry extract

elderberry (Artemis International USA). Moreover, studies with time-lapse video-recording and flow cytometry suggest induction of apoptosis in brain tumour cells *in vitro* (unpublished data).

Grape seed extract: oligomeric proanthocyanidins

Red grape seed extract belongs to the category of proanthocyanidin within the flavonoid super-family. Some of the sub-units found in grape seed extract are also present in chokeberry extract so that both extracts might be expected to have some similarities in their therapeutic potential. Grape seed extract is made up of three monomeric sub-units, namely catechin (Fig. 7), epicatechin (Fig. 8), and epicatechin (-3-0-) gallate (Fig. 9). These subunits can elongate themselves, via a condensation reaction, to become dimers, trimers and oligomers, hence giving rise to their alternative name, oligomeric proanthocyanidins.

Red grape seed extract, like with other flavonoids, has a wide variety of beneficial effects on health. These include potent antioxidant, anti-viral, anti-inflammatory, anti-allergic and anti-atherosclerotic effects properties and endothelial relaxantion [35]. Red grape seed has anti-cancer properties in various peripheral human cancers [1, 2].

Pilot studies in our laboratories indicate that red grape seed extract has anti-invasive properties that are different from those of other flavonoids we have tested. In particular, it influences the expression of certain adhesion molecules (down-regulation of CD44 or cluster of differentiation 44 and up regulation of NCAMs or neural cell adhesion molecules). CD44 is a mediator of glial cell invasion [33], while NCAMs hold the major mass together and are characteristic of neoplastic glia with poor invasive potential [14].

Tomatoes: lycopene

Unlike flavonoids, which are water soluble, carotenoids are lipid soluble. Carotenoids have been studied extensively for their anti-cancer properties in peripheral cancers [48] but not in brain tumours. Lycopene (Fig. 10) is one of 600 carotenoids found mostly in ripe tomatoes, giving them a characteristic red pigmentation. Humans are capable of absorbing and metabolizing over 50 dietary carotenoids [23]. Alpha-carotene, beta-carotene, lutein, lycopene and alpha-cryptoxanthine, are among the most abundant carotenoids in human blood. As far as the mode of action of lycopene is concerned, it has been shown to inhibit proliferation of various types of peripheral cancers including those of lung, breast and endometrium [27]. We have shown that lycopene is able to reduce the motility of brain tumour cells but we have not assessed its anti-proliferative properties.

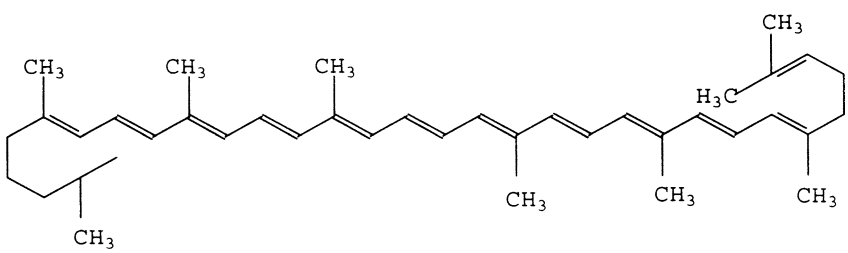

Fig. 10. Structure of the carotenoid, lycopene, found in ripe tomatoes

Selenium

Selenium is a trace mineral that occurs naturally in foods such as whole grain, seafood, brazil nuts, garlic, eggs and mushrooms. Selenium was first associated with protection against cancer in the late 1960s [49]. Knowledge gained since then has clarified the functions of selenium in normal metabolism, has supported a recommended daily allowance (50–70 μg) and has shown that selenium supplementation may prevent cancer in animals. Several hypotheses have been proposed to explain this anti-cancer property:- These include induction of apoptosis and protection against oxidative damage, reflecting the function of selenium as an essential constituent of the antioxidant enzyme glutathione peroxidase. Clark *et al.* [9] conducted a double-blind, placebo-controlled trial to test the hypothesis that a nutritional supplement of selenium, as selenized yeast (200 μg daily) would reduce the occurrence of cancer. Over 10-years a cohort of 1,312 patients was studied by the Nutritional Prevention of Cancer Study Group and the results showed significant reductions in total cancer mortality (50%) and in total cancer incidence (37%).

The trace element selenium is anti-proliferative and induces apoptosis in established human brain tumour cell lines (homogeneous population of cells) [57, 52]. Recent studies in the heterogeneous population of cells in low passage, biopsy-derived glioma cultures showed that selenium reduced cell viability, in a dose dependent manner, and induced apoptosis [25].

Clinical trial: a nutraceutical approach

In complete contrast to their beneficial effects, some flavonoids may also be harmful possibly as a consequence of pro-oxidant rather than antioxidant action [7, 45]. Moreover, although there is much evidence in support of a flavonoid-rich diet for cancer prevention, the conditions and levels of flavonoid intake, that for there to be a risk to health remain uncertain [50], clinical trials must, avoid exceeding the therapeutic doses of a given micronutrient and avoid toxicity.

In the 1990s, a pilot study reported that selenium, when added to the diet of 15 patients with a malignant brain tumour did not prolong postoperative survival [40]. Several studies have assessed the impact of single micronutrients on peripheral cancers with encouraging findings, but none used a combined formulation of micronutrients that can be expected to have additive or supra-additive beneficial effects. Moreover, none of the other agents mentioned in this review have been assessed in clinical trials for malignant intrinsic brain tumours. We plan to initiate a clinical trial entitled ''Modulation of biological behaviour of brain tumours using a multiple micronutrient schedule''. In this the 6 micronutrients discussed above, in doses determined from our laboratory-based research, will be assessed in patients newly diagnosed with glioblastoma multiforme.

Clomipramine in glioma therapy

Conventional approaches to the treatment of intrinsic brain tumours are generally based on the concept of ''cell kill''. There are inherent difficulties in such strategies, because populations of invading neoplastic cells, which are not actively dividing, may be refractory to radiotherapy and also protected from the action of many cytotoxic drugs by being located in an area with a normal blood-brain barrier. Moreover, rather than necrosis, the preferred mode of cell death would be either apoptosis or a combination of the two. Agents with the desired effects in tumour cells, whilst leaving normal brain cells unaffected, may be found in drugs that have a long record of use for other purposes.

One opportunity concerns the role of mitochondria which are beginning to be seen as much more than just the energy centre of the cell. These organelles are heavily implicated in apoptosis, and may contribute to cancer formation and cell death. Invasion by tumour cells is highly energy (ATP or adenosine triphosphate) dependent, but tumour cells frequently show abnormalities in the number, structure and function of their mitochondria, and are more reliant on glycolysis than non-transformed cells. In the 1950's Warburg reported that mitochondrial respiration was decreased in neoplastic tissue, along with a lowering of the cellular complement of mitochondria [53]. This indicates that tumour cells rely more heavily on glycolysis to furnish their ATP, enabling them to exist under hypoxic conditions in which non-neoplastic cells could not survive [29].

A number of studies carried out in the 1970's showed that a variety of heterocyclic compounds showed selective inhibition of mitochondrial function in yeast cells. Thus, the tricyclic compounds act on membranes, particularly those of the mitochondria, inhibiting respiration and leading to limitation of ATP production. This was found to be a common all tricyclic compounds studied [20] but, there seemed to be no clear relationships between chemical structure and pharmacological

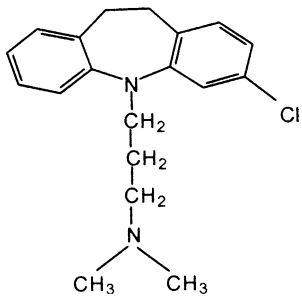

Fig. 11. Chemical structure of the tricyclic anti-depressant, clomipramine

activity. Chlorine derivatives of imipramine and promazine (chlorimipramine and chloropromazine) were, however, more clinically active than the parent molecule [30] and both derivatives were shown to preferentially inhibit mitochondrial function in the intact yeast cell [54].

Chlorimipramine (also known as clomipramine or anafranil) is a drug used in the treatment of obsession and has been in routine clinical use for over 30 years. It belongs to the dibenzazepine class of pharmacologic agents (Fig. 11) known as tricyclic antidepressants. Studies using yeast cells and human cultured fibroblasts showed that clomipramine is strongly lipophilic; it targets the mitochondrion where it partitions into the inner membrane and blocks the respiratory function [55, 20, 30]. Clomipramine also has an advantage over current anti-cancer drugs, which tend to cause irreversible DNA damage, indiscriminately, in both normal and neoplastic cells. It is non-mutagenic and has low toxicity and crosses the blood brain barrier. Some investigations of the effects of tricyclic drugs on various types of cancer cells have been made and a number of studies show that clomipramine is active against drug-resistant leukaemias, renal cancer cells and solid murine tumours [32, 51].

We assessed the effects of clomipramine, clofazimine and chlorpromazine, at different concentrations, on a range of different cell cultures derived from brain tumours [12]. Clomipramine had the most powerful anti-cancer cell effect and this effect was not reversible after a two hour exposure *in vitro*. Further studies showed that clomipramine had a dose dependent, selective cytotoxic effect on all brain tumours that were tested (astrocytomas, mixed oligo-astroctomas, glioblastoma multiforme and meningiomas) whereas normal human astrocytes were unaffected.

To examine the mechanism of action of clomipramine we used a series of mitochondrial respiratory enzyme assays. Mitochondria isolated from the heart, liver, kidney and brain were studied as well as the 12 brain tumour-derived cell cultures described above. The drug inhibited complex III of the respiratory chain, resulting in an elevation of reactive oxygen species, cytochrome c release and caspase-activated apoptosis. In addition, the mitochondria lost their membrane potential, became swollen and died. Furthermore using controllable p53 transfected glioma cell lines, we showed that apoptosis was independent of p53 status [11]. Clomipramine also induces apoptosis in human acute myeloid leukaemic cells [56].

The rationale for the selective response of tumour cells, as opposed to normal brain cells, to clomipramine is based, on the concept that the compromised respiratory function of cancer cell mitochondria leads to apoptotic cell death as opposed to necrosis. Interestingly, it has been recently reported that members of another group of antidepressant drugs, the selective serotonin reuptake inhibitors (SSRIs), which include Prozac^{TM}, may have a similar pro-apoptotic effect on lymphoma cells (Gordon, personal communication).

Cathepsin L, a lysosomal protease, is involved in the invasive cascade of brain tumours and is upregulated with increasing grade of tumour malignancy. It has been shown recently to inhibit the action of pro-apoptotic agents [26]. Studies using antisense transfection showed that cathepsin L inhibition resulted in an enhanced apoptotic response in malignant glioma cells *in vitro* treated with clomipramine [26].

Clinical trial: clomipramine

The data available suggests that clomipramine (but not other heterocyclic drugs studied by our group) may be useful in the treatment of patients with a primary brain tumour of various histological type & grade of malignancy. The drug is essentially non-toxic, inexpensive; it has been in clinical use for some 35 years (for depression & obsessive-compulsive conditions); it crosses the blood-brain barrier; it has a long half-life, sequesters in the brain & lung and, at the appropriate dose, selectively kills tumour cells without damaging the normal cells of the brain. These features make this agent an excellent candidate for clinical initiation in patients with a brain tumour. There are already around 200 ''anecdotal'' cases of patients with a range of different primary brain tumours who are taking clomipramine in the UK. Although this is a highly heterogeneous group in terms of histological diagnosis, site of tumour, age, stage of disease progression and other treatments given,

there have been numerous reports of survival benefit with good quality of life together with evidence from MRI scanning of disease regression [43]. We plan to initiate a formal, rigorous, clinical study in which patients newly diagnosed with anaplastic astrocytoma or glioblastoma multiforme will receive an initial daily dose of 25 mg Anafranil™ (clomipramine/chlor imipramine), escalating to 150 mg in steps at 3-day intervals.

Conclusion

The various lines of experimental investigations reviewed have provided substantial evidence that to anticipate that further clinical research will result in the translation of the proposed treatment strategies – either singly or in combination – into benefit to patients suffering from the devastating group of neoplastic diseases that affect the central nervous system.

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