

## 2-Methoxyestradiol as a Potential Cytostatic Drug in Gliomas?

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**Abstract:** Gliomas of astrocytic origin show only a limited chemotherapy response. Chemoresistance is most pronounced in glioblastoma multiforme, the most common and most malignant glioma, with median survival times not much longer than one year. Failure of chemotherapy partly relies on protective mechanisms against the commonly used DNA alkylating agents, but also on the constitutive activation of the pro-survival PI3K-Akt pathway in glioma cells, which inhibits apoptosis. Therefore, new drugs with an alternative mechanism, independent of DNA alkylation, are required.

The microtubule targeting drug 2-methoxyestradiol (2-ME) efficiently induces mitotic arrest, apoptosis, but also autophagic cell death in glioma cells *in vitro*. Moreover, it may be able to inhibit vascularization of the highly vascular glioblastomas, because the drug influences blood vessel sprouting via a HIF-1-dependent mechanism. Although high doses of i.p. injected 2-ME were recently shown to be effective in an orthotopic rat glioma model, clinical phase I/II trials revealed low oral bioavailability. One of the most exciting future perspectives will be the currently ongoing development of improved 2-ME analogs. Compounds, sulphamoylated at positions 3 and 17, combine sufficient toxicity against tumor cells with resistance against metabolic degradation and sufficient plasma levels in experimental animals. They were found to be superior in some animal models of tumor growth and vascularization, following oral application.

**Key Words:** Glioma, glioblastoma multiforme, chemotherapy, vasculature, 2-methoxyestradiol.

### 1. INTRODUCTION

#### 1.1. Glioma Epidemiology and Etiology

Gliomas are relatively rare human cancers, originating from glial cells (astrocytes or oligodendrocytes) of the central nervous system (CNS). They can affect all parts of the CNS, but occur most frequently in the brain. Astrocytic tumors generally exhibit diffuse infiltration of brain tissue, although they do not distribute metastases to other body sites outside the CNS. Despite its infiltrative growth, the least malignant subgroup of adult astrocytic tumors, the diffuse astrocytoma (WHO grade 2), may grow undetected for years without causing seizures or other neurological symptoms. However, it bears an intrinsic risk of progression to faster growing anaplastic astrocytomas (WHO grade 3) or to a so called 'secondary' glioblastoma multiforme (GBM, WHO grade 4). It should, however be stated that the vast majority of GBM cases does not exhibit clinical evidence for an earlier precursor lesion and is thus termed 'primary GBM'. Glioblastomas can rapidly compress vital brain structures, increase intracranial pressure to critical values or cause massive brain hemorrhages. The tumor usually causes death of the patient within a short period after diagnosis. GBM is the predominant adult brain tumor with an incidence rate of 3.55 new cases per 100,000 population per year in Switzerland and 2.96 per 100,000 population per year in the USA [1]. The age-adjusted incidence rates of brain tumors in general, including GBM, tend to be highest in developed, industrial countries, and Caucasians are more frequently affected than people of Asian or African descent [2]. It is unknown to which extent these differences may reflect the socio-economic status or true differences in genetic susceptibility.

A huge number of environmental carcinogens, most often in a setting of occupational exposure, have been implicated in glioma carcinogenesis by epidemiological studies (for review see [2]). However, the results are often not confirmed in other populations. In addition, some studies suggest, but do not prove, a causal relationship between parental preconceptional carcinogen exposure and

brain tumor incidence of the offspring. The suspected noxes included formaldehyde, vinyl chloride, polycyclic aromatic hydrocarbons, some insecticides, lead, arsenic compounds, mercuric compounds and petroleum products. Although various chemicals were found to increase the incidence of gliomas in experimental animals, there is no concomitant evidence that this applies to humans. This lack of evidence also concerns N-nitroso compounds and their precursors, some of which were potent brain tumor inducers in animals. Several studies analyzing possible associations between brain cancer and nitrosoamines or nitrates from human diet sources revealed conflicting results [2]. The only environmental factor which unequivocally increases brain tumor risk in humans is therapeutic X-irradiation, especially in young children [3, 4]. Several rare autosomal dominant disorders are associated with multiple tumors, including gliomas, thus supporting the role of genetic predisposition in glioma carcinogenesis. A fraction of these familial cancer syndromes (reviewed in [2]) are caused by germline mutations of the TP53 tumor suppressor. In such families, 14% of all neoplasms are brain tumors, 64% of the latter being of astrocytic origin. This is of interest, because TP53 mutations are also frequent events in sporadic 'secondary' GBM. This suggests that early TP53 inactivation may at least be a molecular mediator of tumor development in a subset of GBM cases. For a short review of other molecular pathways altered in GBM see [1].

#### 1.2. Low Efficacy of Alkylating Chemotherapy in the Last Three Decades

Chemotherapy represents a major challenge in anaplastic astrocytomas and especially in GBM. The highly infiltrative nature of these tumors does not allow a complete surgical removal of all dispersed tumor cells which will cause a recurrence. To retard recurrence or progression to GBM, radiotherapy and chemotherapy are required. The median survival times of newly diagnosed GBM patients, treated according to today's standards, are not much longer than one year [5] or are even shorter for the worst subgroup [6].

The three decades preceding the introduction of the alkylating drug temozolomide (TMZ), have seen a heated controversy about the efficacy of chemotherapy. Since the uncertain results of randomized clinical trials in high grade gliomas had elicited a controversial discussion of that issue, the clinical practice varied both,

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within a country and internationally. In 2002, the international Glioma Meta-analysis Trialists (GMT) Group summarized and compared twelve randomized trials, including a total number of 3004 patients who suffered from all types of anaplastic gliomas and GBM. The chemotherapy regimens in these studies had been based on DNA alkylating nitrosoureas (mostly BCNU or CCNU) but sometimes included other alkylating agents, such as procarbazine, dacarbazine and mitolactol, topoisomerase-II inhibitors (epipodophyllotoxin) or microtubule targeting agents (vincristine). Regarding overall survival, the established chemotherapy regimens at that time were found to have a small, but significant, effect which did not depend on tumor histology [7]. The mean 1- and 2-year survival rates of patients with anaplastic astrocytomas increased from 58% to 63% and from 31% to 37%, respectively. The corresponding increases for GBM patients were 35% to 41% and 9% to 13%, respectively. As mentioned by the authors, this small benefit had to be offset by the individual patient and his family against the background of a deterioration of life quality, which may be caused by chemotherapy. Due to the inhomogeneous and incomplete assessment of life quality in the trials, the effect of chemotherapy on this parameter could not be judged on a rational base in a meta-analysis. At that time, the small objective survival benefit justified adjuvant chemotherapy with nitrosoureas as a standard, according to some clinical trialists, while others considered radiotherapy alone to be appropriate.

### 1.3. Meaningful, But Limited Efficacy of Temozolomide in the Treatment of GBM

During the era of nitrosoureas, the median survival time of GBM patients was below one year from the time of diagnosis [7]. Besides limited bioavailability of the drugs in the CNS, specific mechanisms of chemoresistance at the cellular level have been identified and implicated in the insufficient chemoresponsiveness. At least one of these factors, methylguanine-DNA-methyltransferase (MGMT), which removes alkyl residues from the O<sup>6</sup>-position of guanosines, was demonstrated to play an important role in GBM resistance to alkylating agents. A low level of MGMT mRNA expression or epigenetic silencing of the gene by promoter methylation [8] was found to be a favourable prognostic marker, suggesting a better chemotherapy response. The newly introduced temozolomide (TMZ) is able to suppress MGMT activity [9], a feature which may be favourable in GBM [8].

Recently, the significant increase in overall survival of newly diagnosed GBM patients by concomitant and adjuvant TMZ was proven in a phase-III multicenter study [5]. The authors compared 286 GBM patients receiving tumor resection and fractionated 60 Gy radiotherapy with a group of 287 patients additionally receiving TMZ concomitant with radiotherapy, followed by adjuvant TMZ for up to 6 months. The drug improved median survival by 2.5 months, and the long term increase in overall survival (25.6 % after 2 years) became meaningful, as compared with the minimal long term effects reported earlier for nitrosoureas [7].

Mirimanoff and colleagues [6] performed a retrospective analytical refinement of this trial by applying the recursive partitioning analysis [10] to define three prognostic subgroups of GBM patients according to age, Karnofsky performance status (KPS) and mental status. Applying this grouping, the authors showed that the 2-year survival benefit for the two more favourable subgroups remained highly significant ( $p < 0.0001$ ), while TMZ benefit was of borderline significance ( $p = 0.054$ ) for GBM patients older than 50 years, who exhibited either a KPS <70 or a lower mental status (inhibiting the ability to work).

Taken together, these results suggest that TMZ may have improved the long term outcome of adjuvant chemotherapy in a meaningful way, and that the benefit may be especially pronounced in

GBM patients with lower MGMT-based chemoresistance. The clinical studies show, however, that the results of classical alkylating chemotherapy remain unsatisfactory, and the development of drugs with an alternative mechanism is required.

### 1.4. Tumor Vasculature as a New Therapeutic Target

The important role of tumor vasculature may open an entirely new field of anticancer drug development [11], because tumor growth beyond a diameter of some millimeters requires neovascularization [12]. High grade gliomas, and especially GBM, are highly vascular and contain newly built blood vessels, to ensure sufficient nutrient and oxygen supply. Some efforts have thus been spent in trials to inhibit glioma growth indirectly by attacking the vasculature.

As thalidomide inhibits angiogenesis [13], it was used in an early phase-II trial in high grade gliomas as a single agent [14], where it exhibited limited efficacy. The response was enhanced, when a nitrosourea was applied in parallel with thalidomide in a second phase-II trial [15], thus supporting the strategy of a combined action of an alkylating agent and an angiogenesis inhibitor. Later, more specific approaches have been elucidated, with the intention to block specifically the tyrosine kinase activity of the receptor for the growth factor VEGF (*vascular endothelial growth factor*), which is expressed in gliomas [16, 17] and induces neoangiogenesis in the hypoxic areas. The oral VEGFR-tyrosinekinase-inhibitor Vatalanib, applied as a single agent, induced some response in terms of progression-free survival (PFS) in patients with recurrent GBM [18]. PFS time increased if Vatalanib was combined with either TMZ or a nitrosourea [19]. The most recent development in this field is a direct antibody attack against the growth factor VEGF. A functional IgG1 antibody had been originally developed as a murine monoclonal by Ferrara and coworkers [20] and was later humanized to destroy its immunogenic potential in humans. Meanwhile, the humanized form, Bevacizumab, was used in trials for various human cancers. Due to successful phase-III trials, it received FDA approvals for the use in metastatic colorectal and metastatic non-small-cell lung cancer. The antibody was recently also tested in a first phase-II trial in recurrent malignant glioma, which included 72% of GBM cases [21]. In that study, Bevacizumab was not applied as a monotherapy, but together with the topoisomerase-I inhibitor Irinotecan. A control group treated only with Irinotecan was not included, which limits the interpretation of antibody efficacy. Nevertheless, the results showed that the regimen was generally active and that the benefit in terms of PFS in GBM patients exceeded that reported earlier for a combination of Vatalanib and alkylating agents [19]. A similar phase II study of combined antibody and Irinotecan application demonstrated activity of the treatment in recurrent GBM [22]. Taken together, tumor vasculature as a therapeutic target seems to be a hopeful supplementation of strategies, which solely attack glioma cells at the DNA level.

As will be shown in the following sections, the development of the steroid drug 2-methoxyestradiol (2-ME) and its analogs is of interest regarding gliomas for several reasons: a) 2-ME has pleiotropic anti-tumor activities, that include both, inhibition of angiogenesis and cytotoxic effects on tumor cells, b) its mechanism does not rely on DNA alkylation and is thus not likely to be affected by MGMT, c) the drug may induce autophagic cell death in glioma cells, which resist apoptosis, d) it was recently shown to be effective in an orthotopic rat glioma model, e) it mostly exhibited moderate side effects in clinical phase I/II studies of GBM and other malignancies and f) analogs with increased oral bioavailability were recently synthesized and were demonstrated to be potent inhibitors of tumor growth and neoangiogenesis in animal models, following oral application.

## 2. 2-METHOXYESTRADIOL: A NATURAL MAMMALIAN STEROID WITH PLEIOTROPIC ANTI-TUMOR EFFECTS IN PRECLINICAL MODELS

2-methoxyestradiol (2-ME, Fig. 1) is a natural product of mammalian steroid metabolism, which is formed by sequential hydroxylation and O-methylation of the estradiol molecule at the 2-position. It is known to exhibit only low estrogen receptor binding [23], and thus to possess only minimal hormonal activity. A little more than one decade ago, it was suggested to be merely a metabolite without any significant biological activity. However, in 1994, D'Amato and colleagues [24] detected the ability of 2-ME to bind to the colchicine site of  $\alpha$ -tubulin and to inhibit microtubule polymerization. The steroid became an interesting new candidate drug to influence both, mitotic spindle apparatus of dividing cells and cell motility by disturbing the dynamics of the microtubule cytoskeleton. In the following decade, a large spectrum of anti-tumor activities was described in cell cultures and animal models (for review see [25]).

Induction of apoptosis in tumor cells by micromolar drug concentrations, as well as an *in vivo* efficacy of 2-ME in a murine xenograft model, have been demonstrated for the first time in the multiple myeloma [26]. The study revealed also a lower VEGF secretion and a lower microvascular density in the xenografts of treated animals, suggesting an influence of 2-ME upon VEGF-induced vascularization. In the same year (2002), 2-ME was shown to induce apoptosis in an extended panel of multiple myeloma cell lines and in patient samples. Moreover, total tumor regression was reached in the murine xenograft model, which was maintained for three weeks after cessation of the therapy [27]. At that time, a pathway connecting the  $\alpha$ -tubulin interfering properties of 2-ME with apoptosis induction and VEGF interference was not known. Results derived from pancreatic tumor cells [28] showed G<sub>2</sub>/M arrest of the mitotic cycle in some, but not all, treated cell lines. It occurred together with mitochondrial apoptosis and phosphorylation of the protein bcl-X<sub>L</sub>, one of the anti-apoptotic members of the bcl-2 family. A possible sequence of events, connecting 2-ME-induced microtubule dysfunction, G<sub>2</sub>/M arrest and induction of intrinsic, mitochondrial apoptosis could be suggested to occur in some of the cell lines tested.

In search for 2-ME-regulated genes, a first microarray approach in multiple myeloma cells revealed 2-ME-induced transcriptional upregulation of some pro-apoptotic and ROS (*reactive oxygen species*)-generating proteins. The latter result, together with inhibition of manganese and copper/zinc-dependent superoxide dismutases [29], may explain the observed ROS induction by 2-ME in several tumor cell lines, which may further support mitochondrial apoptosis. In cell lines of various tumor types, an upregulation of death receptor 5 (DR5) was reported [30], suggesting an additional role of the receptor-mediated, extrinsic apoptotic pathway in 2-ME toxicity. In recent years, 2-ME was shown to exhibit anti-proliferative and cytotoxic properties in a large number of cell lines of multiple human tumor types, including colorectal carcinoma [31], pancreatic carcinoma [28, 32], osteosarcoma [33, 34], melanoma [35], Ewing's sarcoma [36], medulloblastoma [37] and glioma [37, 38, 39, 40, 41]. Besides other animal models, 2-ME efficacy was recently demonstrated in a xenograft model of metastatic and osteolytic breast cancer [42], in which the drug showed activity not only

against the tumor cells in the soft tissue, but also against tumor cells in the bones.

## 3. INSIGHTS INTO THE MECHANISM OF 2-METHOXYESTRADIOL INTERFERENCE WITH VEGF-SIGNALLING, APOPTOSIS AND CELL CYCLE

The first recognized feature of 2-ME was its ability to bind at or near the colchicine site of  $\alpha$ -tubulin and to disturb microtubule polymerization [24]. It has been reported that therapeutic drug concentrations disrupt interphase and mitotic microtubules and cause mitotic arrest. These concentrations also inhibited the expression, nuclear accumulation and transcriptional activity of HIF-1 $\alpha$  [43]. This downregulation of the main hypoxia-inducible transcription factor was suggested to be a downstream effect of tubulin interference, because several microtubule-depolymerizing drugs also inhibited HIF-1 $\alpha$ , while other inducers of mitotic arrest did not. This hypothesis offered a first link between the microtubule activity of 2-ME and its microvascular effect, because HIF-1 $\alpha$  is the main regulatory element, required to detect tumor hypoxia and to induce VEGF expression. Under hypoxic conditions, the rate of HIF-1 $\alpha$  degradation is diminished, thus favouring the formation of a heterodimeric transcription factor. The latter binds to the RCGTG hypoxia response element and induces transcription of several hypoxia response genes, VEGF being among them. HIF-1 $\alpha$  inactivation thus inhibits the sprouting of new blood vessels into the tumor. Even the above mentioned phosphorylation of an anti-apoptotic member of the bcl-2 protein family [28] is a feature shared by other microtubule-disrupting drugs. Tubulin binding has thus been regarded as the common cause of anti-proliferative, pro-apoptotic and microvascular effects of the drug.

However, the role of microtubule disruption in the antitumor activity is controversial, because the minimal 2-ME concentrations leading to mitotic arrest, do not destroy mitotic spindles [44]. A recent study showed in detail, that the drug concentrations required for efficient inhibition of tubulin polymerization were indeed far beyond those required for efficient mitotic arrest in the breast cancer cell line MCF-7 [45]. Using purified tubulin, the maximal reduction of the polymerization rate *in vitro* (60%) was reached for 200  $\mu$ M 2-ME. Even significantly higher concentrations were needed to depolymerize normal MAP-containing microtubules, while the IC<sub>50</sub> of mitotic arrest was 1.2  $\mu$ M. Using microscopical video techniques, the authors measured the concentration-dependent microtubule dynamics *in vitro* and *in vivo*. For the latter purpose, they used fluorescence time lapse imaging of cells, which expressed tubulin, labeled with GFP (*green fluorescent protein*). *In vitro*, the growth and the shortening rates ( $\mu$ m/min) were slightly diminished at 4  $\mu$ M 2-ME, but the percentage of time, which the microtubules spent in the "paused state", was significantly enhanced. The inhibition of dynamics may be much more important at low micromolar 2-ME concentrations as compared with a loss of existing microtubules per se. This view was supported by the same type of analysis performed *in vivo* at the IC<sub>50</sub> of mitotic arrest. Moreover, in cultures incubated with up to 10  $\mu$ M 2-ME, the cytoskeleton of interphase cells was similar to that of controls. The microtubules emanated from a central organizing center, were well spread and not bundled or contorted. The morphology of most mitotic spindles was bipolar at the IC<sub>50</sub> of mitotic arrest, but one or more uncongressed chromosomes were often located at the poles, giving rise to a stronger aster at this pole. At 10  $\mu$ M, most spindles were not bipolar, but contained many tiny asters. These results suggest, that inhibition of microtubule dynamics may be the primary cause of mitotic arrest. The mechanism, however, was not fully understood at this point.

New insights were revealed by a recent study, which compared the effects of 2-ME in two clones of breast carcinoma cells (MDA-MB-435), one of which contained two  $\alpha$ -tubulin mutations [46], rendering this clone largely resistant to the drug, according to pro-

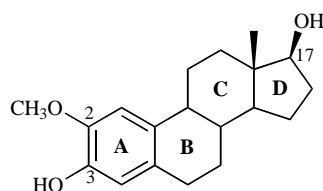


Fig. (1). 2-Methoxyestradiol.

liferation assays ( $IC_{50}$  of 99.5  $\mu$ M versus 1.3  $\mu$ M in the parental cells). The study focused mainly on the question of whether or not  $G_2/M$  arrest is a tubulin-dependent event, and whether molecular pathways unknown to date may play a role. At first, the authors confirmed that in the low micromolar range 2-ME induced  $G_2/M$  arrest selectively in the parental cells, an effect which became irreversible after prolonged incubation at 5  $\mu$ M, and was coincident with enhanced apoptosis. Next, they intended to find possible new molecular pathways by applying a gene array analysis and identified 264 genes, which were differentially regulated by 2-ME. The most interesting result was an increase in some cell cycle regulators involved in the control of the mitotic spindle assembly checkpoint, such as MAD2, BUB1 and CDC20.

The anaphase promoting complex (APC) plays an important role in the transition from metaphase to anaphase [47] and is a direct target of MAD2, the enhanced expression of which inhibits APC function. To find out whether 2-ME induces mitotic arrest by APC inhibition, two direct targets of this complex were analyzed in more detail, securin and cyclin B1. Securin inhibits sister chromatid separation, and proteolysis of both proteins is required to initiate the transition to anaphase. APC, which acts as a ubiquitin ligase, is required to label securin and cyclin B1 for subsequent proteolysis. 2-ME enhanced the amounts of both proteins in a tubulin-dependent manner, since the enhancement was less pronounced (securin) or even absent (cyclin B1) in the tubulin mutant clone. The study excluded APC inhibition via direct 2-ME binding to the complex, using an *in vitro* assay. Although the precise mechanism of the tubulin mutations in that model is not clear, it seemed reasonable to conclude from these experiments that APC inhibition is a possible molecular mechanism of 2-ME-induced mitotic arrest.  $G_2/M$  arrest, meanwhile demonstrated in many tumor cell lines, may also play a role in the radiosensitizing effect of 2-ME observed in lung cancer, colon carcinoma and prostate cancer cells [48, 49, 50], and recently described in two glioma cell lines [40].

HIF-1 $\alpha$  inhibition by tubulin dependent mechanisms [43] has gained further probability by an additional study, which confirmed that several, clinically relevant microtubule disrupting agents, such as vincristine, taxotere, epothilone B, discodermolide, 2-ME and colchicine, all decrease HIF-1 $\alpha$  protein levels and transcriptional activity [51]. HIF-1 $\alpha$  mRNA levels were not significantly reduced, suggesting a mechanism acting on the protein at the translational or post-translational level. Further experiments suggested that translation itself is most likely inhibited. Some hints were discussed that HIF-1 $\alpha$  translation may depend on mRNA coupling to microtubules. The link between cytoskeleton and regulation of hypoxia response was further supported by comparisons of an ovarian cancer cell line with an epothilone B-resistant subclone. While the drug downregulated HIF-1 $\alpha$  protein and impaired its nuclear accumulation in the parental line, this was not the case in the resistant subclone.

#### **4. MECHANISM OF 2-METHOXYESTRADIOL: WHAT IS KNOWN TO OCCUR IN GLIOMA CELLS?**

In the last five years, a few studies analyzed 2-ME in glioma cell cultures. Morphological alterations, such as retraction of cell processes and rounding up, were commonly observed. A filigran microtubule network was still visible in confocal micrographs of completely rounded interphase cells after prolonged incubation with high 2-ME concentrations [39], far beyond the level needed for efficient growth inhibition and cell death induction. This was in accordance with the above discussed observations in breast cancer cells [45], indicating that no gross changes of microtubule cytoskeleton are required to induce  $G_2/M$  arrest and to transmit the death signal.

Profound growth inhibition at low micromolar concentrations, applied for several days, was found in all studies and usually measured using microtiter tetrazolium (MTT) assays. Although mainly

focusing on medulloblastoma, Kumar and colleagues [37] reported a 38% decline in viable cell number (MTT), when T98G glioma cells were incubated with 2  $\mu$ M 2-ME for three days. Applying 10  $\mu$ M for four days, Lis et al [38] found a marked decrease in viable cells between 60 and 90 % for the human glioma lines U87MG, U138MG and T98G in the same assay. As the latter two lines carry TP53 mutations, these results suggest that inactivation of the tumor suppressor may play no major role for 2-ME sensitivity, although TP53 protein expression was found to be induced by 2-ME in the wild type line. Independency from TP53 status is a finding of some therapeutical significance, since TP53 is mutated in many gliomas, is believed to play a major role for the genesis of secondary glioblastoma and plays a vital role in the regulation of cell cycle and apoptosis. Quite similar results were obtained in our laboratory for the lines U87MG, U138MG, LN405 and the rat glioma RG-2 [39], demonstrating a marked response to 2  $\mu$ M 2-ME, applied for 6 days.

Morphological evidence for apoptosis was consistently reported in the form of fragmented nuclei and apoptotic bodies, visible in Hoechst 33258 stains [38, 39, 41]. Micronucleated cells indicated a hampered mitotic division [38]. All nuclear alterations were independent of TP53 mutational status of the cell lines tested. Interestingly, no corresponding morphologic alteration in the nuclei of 2-ME-treated adult rat astrocytes was observed [38], indicating drug specificity for astrocytic tumor cells. We demonstrated that even short term 2-ME treatments resulted in marked responses [41], suggesting fast apoptotic induction. Usually a 12 h incubation with 2  $\mu$ M was sufficient to achieve a statistically significant decrease in viable cell number. 20  $\mu$ M induced substantial levels of fragmented nuclei after 24 h. The apoptotic nature of glioma cell death, or at least a participation of apoptosis in cell death, was further supported by the observed induction of the effector caspase 3 [30, 39] and other caspases [30] in human and rat glioma lines.

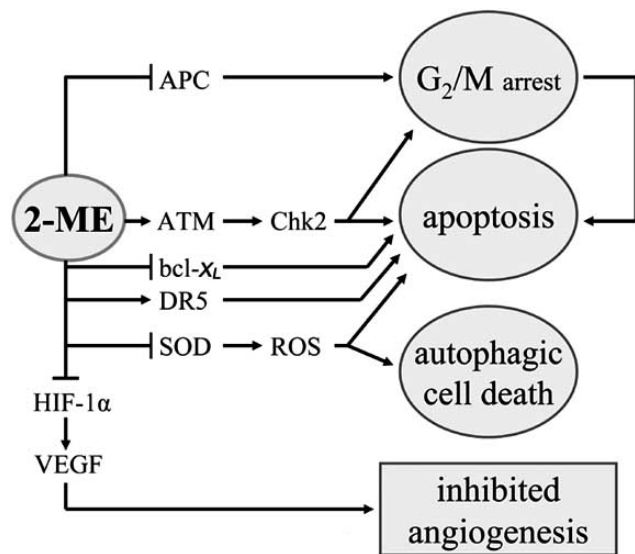
Besides intrinsic or mitochondrial apoptosis [28], the extrinsic, receptor-mediated pathway had also been implicated in 2-ME toxicity in a single study, which included glioma cells in some experiments [30]. For the breast cancer cell line MDA-MB-231 and human umbilical vascular endothelial cells (HUVECs), a profound synergistic effect of 2  $\mu$ M 2-ME and 50 ng/ml TRAIL was found in cell viability assays [30]. The authors explained this result by moderately enhanced expression of DR5 (death receptor 5) protein in the plasma membranes of 2-ME-treated cells, which they detected by Western blotting. It was associated with a parallel increase in apoptosis induction, as judged by the 85 kD PARP fragment. Exploiting cleavage of specific fluorogenic substrates, the authors documented early simultaneous peaks of caspase-8 and -9 activities in MDA-MB-231 cells at a time after stimulation, at which caspase-3 activity was still increasing. The early activation of caspase-8 is quite suggestive of an extrinsic apoptotic mechanism to participate in 2-ME-induced cell death. It does not exclude a parallel apoptosome activation by mitochondrial membrane rupture and cytochrome c release (mitochondrial apoptosis). While all other experiments of that study [30] were not performed with glioma cells, the Western blots included the line U87MG, demonstrating a small DR5 protein increase (1.3 to 1.5-fold) following 2-ME administration. Western blot data alone were not conclusive for a DR5-mediated apoptotic mechanism to occur in glioma cells. In our laboratory, no functional synergism between 2-ME and TRAIL was detected by MTT assays in four glioma lines, including U87MG. In addition, no DR5 mRNA induction by 2-ME was detected in Real-Time-PCR experiments [41], suggesting no major role of the DR5 pathway in these cells.

With respect to cell cycle changes, results of some flow cytometric measurements are consistent with  $G_2/M$ -arrest under low micromolar concentrations [38, 40], reported earlier for many tumor cell types of non-CNS origin. However, the fraction of  $G_2/M$ -arrested cells induced by the same drug concentration varied re-

markably among glioma cell lines [38], as reported earlier for pancreatic tumor cells [28].

In addition to the above cited data, the potential value of 2-ME as a radiosensitizing drug in gliomas was recently analyzed in the lines T98G and U251G by Zou and colleagues [40]. This is an interesting aspect because GBM is routinely administered to radiotherapy and concomitant chemotherapy, but glioma cells often exhibit low radiosensitivity. 1 or 2  $\mu\text{M}$  2-ME significantly reduced the surviving fraction of cells irradiated with 2 to 6 Gy of X-rays in a dose-dependent manner, according to the results of a clonogenic assay. The drug enhanced the apoptotic effect of irradiation in glioma cells, but not in lymphocytes. Although 2-ME alone did not elicit a significant degree of DNA damage, it strongly enhanced X-ray-induced DNA damage. Since recruitment and activation of the protein ATM (ataxia teleangiectasia-mutated), a kinase of the DNA damage response pathway, are involved in cell cycle progression and apoptosis, the authors hypothesized that activation of this pathway may be involved in 2-ME-enhanced  $G_2/M$ -arrest and apoptosis. They found unchanged amounts, but increased phosphorylation of ATM and its direct target Chk2.

Figure 2 summarizes the main pathways, which were found to mediate the pleiotropic effects of 2-ME in glioma cells, other tumor cells and HUVECs. It includes the recent discovery that the drug can elicit an alternative cell death pathway in glioma cells, which are resistant to apoptosis (see below).



**Fig. (2).** Diagram showing the main routes of antitumor and antiangiogenic 2-ME effects determined in various cell culture models. 2-ME may elicit the intrinsic, mitochondrial pathway of apoptosis, in which release of proapoptotic proteins from the mitochondria leads to activation of the apoptosome and finally of caspase-3. This mode of apoptosis may be triggered by phosphorylation of the antiapoptotic regulator bcl-X<sub>L</sub>, by activation of ATM kinase and its downstream targets, or by an increase in reactive oxygen species (ROS). The latter process may be explained partly by Mn-SOD and Cu/Zn-SOD inhibiting properties of 2-ME. In cells of low apoptotic sensitivity, such as primary glioblastoma, ROS increase may trigger the autophagic mode of cell death as well. In addition, extrinsic apoptosis via DR5-upregulation may occur in some cell types. Inhibition of the anaphase promoting complex (APC) was recently shown to be a possible mechanism of 2-ME-mediated  $G_2/M$  arrest. Blood vessel sprouting into hypoxic tumor areas is thought to be blocked by the well documented inhibition of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) at the protein level, which, in turn, decreases VEGF-signaling to endothelial cells.

## 5. AUTOPHAGY OVERCOMES APOPTOSIS RESISTANCE IN GLIOMA CELLS

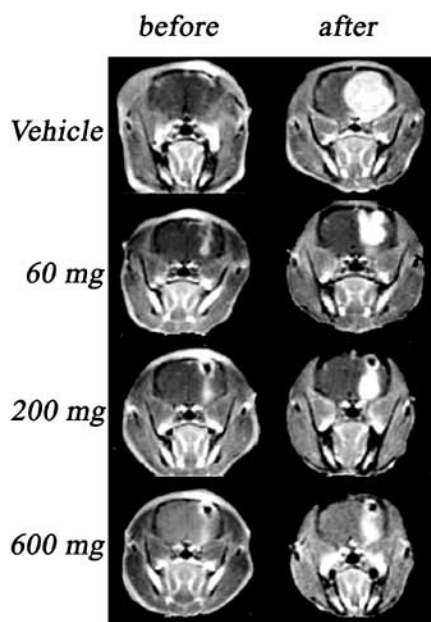
Activation of prosurvival PI3K-Akt signaling pathways and a resulting lower ability to undergo apoptosis are associated with higher tumor grade in gliomas [52]. In GBM, especially in the large group of primary GBM [1], PI3K-Akt signaling may be enhanced by an amplified, overexpressed and/or mutated EGFR (*epidermal growth factor receptor*) or by mutation of the PTEN tumor suppressor, which normally inhibits this pathway. In addition, the transcription factor NF- $\kappa$ B, which is indirectly targeted by Akt, is constitutively upregulated in a fraction of GBM [53]. These enhanced prosurvival pathways render GBM cells less sensitive to apoptosis [54, 55, 56]. Drugs stimulating the process of autophagy of cytoplasmic organelles to levels, which become cytotoxic, may overcome resistance to proapoptotic compounds. TMZ, the current gold standard of GBM chemotherapy, acts mainly as a proautophagic drug in glioma cells [57, 58, 59], although apoptosis can participate in cell death [60]. This proautophagic property may be one of the major advantages of TMZ, besides the already mentioned MGMT suppression. The susceptibility of glioma cells (U87MG, T98G) to proautophagic drugs was further supported by their sensitivity to the mTOR inhibitor rapamycin [59], because mTOR is believed to be involved in autophagy.

A recent study revealed that 2-ME is proautophagic in glioma cells, but not in mouse astrocytes. This feature seems to be related to the ROS inducing property of the drug [61]. Both H<sub>2</sub>O<sub>2</sub> and 2-ME induced autophagy besides apoptosis in various tumor cell lines, including U87MG. Blocking of autophagy inhibited cell death, while cells responded to blocked apoptosis with a shift to the autophagic mode of cell death. Since inhibition of autophagy did not alter the 2-ME-induced ROS production, the latter seemed to be the upstream event. This newly recognized feature of 2-ME may be of additional interest for glioma chemotherapy.

## 6. ACTIVITY OF 2-METHOXYESTRADIOL IN AN ORTHOTOPIC RAT GLIOMA MODEL

Until recently, only the above cited cell culture studies had demonstrated the anti-proliferative, pro-apoptotic and proautophagic effects of micromolar 2-ME concentrations in gliomas. The *in vivo* anti-tumor effect of high doses of 2-ME, i.p. injected as a monotherapy, was proven for the first time in a rat orthotopic glioma model by Kang and colleagues [62]. A high number of 9L rat glioma cells (50,000) was stereotactically implanted into the brains of Fischer 344 rats with established methods [63], giving rise to fast growing brain tumors. The 2-ME treatment in this model turned out to be clearly successful, although the 9L cell line revealed a rather low 2-ME sensitivity, as concluded from proliferation assays. These assays had shown a growth reduction around 50% for a 50  $\mu\text{M}$  concentration under normoxic and hypoxic conditions. To allow *in vivo* bioluminescence imaging (BLI) of HIF-1 transcriptional activity in the growing tumors, the 9L cells had been stably transfected with a HIF-1 responsive luciferase vector. Beginning with day 8 after tumor cell implantation, 2-ME (0, 60, 200 or 600 mg/kg body weight) was applied daily by i.p. injection at 9 consecutive days. Before the therapy was initiated, the substrate for BLI (luciferin) was applied via a single i.p. injection. All treatment groups consisted of 6 animals, and the MRI measured tumor size, prior to the treatment, did not differ significantly between treated and control groups.

T<sub>2</sub>-weighted contrast enhanced MRI revealed a massive and significant, dose-dependent inhibition of tumor growth by 2-ME (Fig. 3 and Table 1). Even for the lowest dose used, a significant ( $p < 0.01$ ) 4-fold reduction of tumor size occurred. Some drug-related side effects were observed for the highest concentration (600 mg/kg body weight), most frequently diarrhea and weight loss (12-15%). One out of six animals in this group died during treatment. The dose-dependent blockage of tumor growth was confirmed by his-



**Fig. (3).** Representative Gd-DTPA-enhanced brain MRI scans of each treatment group before treatment (day 8 after tumor cell injection) and after 2-ME treatment for 9 consecutive days, modified according to reference [62].

tologic tumor volume estimation, based on H&E staining of serial brain slices at the end of the experiment.

Imaging of HIF-1 transcriptional activity (BLI) revealed a 2-ME-induced signal decrease. For doses up to 200 mg/kg, this decrease may simply be due to the correspondingly lower tumor mass in the treated groups, since no significant effect remained after normalization of BLI data to tumor volumes, estimated by MRI. According to BLI data alone, it seemed that 2-ME did not appreciably affect HIF-1 transcriptional activity in this dose range. In contrast to these results, immunohistochemistry of histological brain slices revealed a decrease in gross HIF-1 $\alpha$  protein levels in the drug-treated tumors, in a dose-dependent manner (Table 1). Pimonidazole, a compound binding preferentially to cells with low oxygen tension (<10 mm Hg), labeled a smaller fraction of tumor cells in the treatment groups, according to immunohistochemical staining of pimonidazole binding (Table 1). This result indicated a lower degree of hypoxia in the treated tumors, which is not in accordance with an inhibited neovascularization. However, reduced pimonidazole staining may simply reflect growth inhibition, leading to smaller tumors with better oxygen supply. The combined results of

BLI for the highest dose and of HIF-1 $\alpha$  stains may suggest an antiangiogenic effect of 2-ME in the model.

To determine if the glioma growth inhibition observed *in vivo* was correlated with 2-ME effects on microtubules, the level of acetylated  $\alpha$ -tubulin was analyzed. Most  $\alpha$ -tubulins are known to be acetylated at their N-terminus, a post-translational modification that stabilizes microtubules [64]. Acetylation decreased significantly with 2-ME treatment in a dose-dependent manner (Table 1). This result demonstrated that drug concentrations affecting microtubules must have been reached in the tumor tissue, but does not prove microtubule-dependence of the anti-tumor effect.

Short term treatment with extremely high doses of 2-ME, exceeding the limits of tolerable side effects, is certainly not comparable with the requirements in human chemotherapy. Moreover, the chosen intraperitoneal route of drug administration may favour blood brain barrier passage in the rat model. In human glioma patients, the drug is not primarily intended as a single agent, but rather as a long lasting supplementation of adjuvant chemotherapy, applied in safe doses orally. Nevertheless, the described rat model clearly demonstrated the principal activity of the drug against gliomas *in vivo*.

## 7. LOW SYSTEMIC TOXICITY, BUT INSUFFICIENT BIOAVAILABILITY IN CLINICAL STUDIES

The results of previous clinical studies investigating human tumor diseases showed that the drug was well tolerated in daily doses up to six grams for long time periods, but oral bioavailability was generally low.

A phase I study of locally recurrent or metastatic breast cancer [65] evaluated orally applied 2-ME either as a monotherapy or combined with docetaxel. While the combined treatment resulted in a 20% overall response rate, the monotherapy trial with 31 women did not reveal any objective responses to 2-ME alone. In that trial, either 200 – 1000 mg had been applied once daily or 200 – 800 mg twice daily until progression. Only few patients experienced grade 3 or 4 toxicities, most frequently fatigue, in the 2-ME monotherapy group during a treatment of up to 252 days. Only one woman experienced grade 4 anemia. Increased transaminases or symptoms suggesting significant estrogenic exposure were not observed. In most cases, toxicity was attributed to the underlying malignancy.

Since drug exposure in the dose range tested was suggested to be insufficient, a phase I trial in patients with solid tumors was initiated with the primary endpoints of determining the maximum-tolerated dose (MTD) of orally applied 2-ME capsules, and of further determining the side-effect profile of the drug [66]. Twenty patients of both sexes, suffering from refractory cancers of prostate, ovary, breast, colon, rectum, kidney or adrenal gland, from melanoma or sarcoma, participated in the study. Dose escalation from

**Table 1.** Effects of i.p. Injected 2-methoxyestradiol in a Rat Orthotopic Brain Tumor Model

	Tumor Volume by MRI (mm <sup>3</sup> )	HIF-1alpha Relative	Pimonidazole Relative	Acetylation Relative
Vehicle	283.25 ± 49.87	1	1	1
60 mg/kg	70.21 ± 36.12	0.76 (p = 0.005)	0.73 (p = 0.008)	0.77 (p = 0.025)
200 mg/kg	56.81 ± 26.80	0.67 (p = 0.001)	0.61 (p = 0.002)	0.62 (p = 0.004)
600 mg/kg	12.61 ± 8.03	0.54 (p = 5e-6)	0.45 (p = 9e-7)	0.42 (p = 2e-5)

The tumor volumes of all treatment groups differed significantly amongst each other (p = 0.019 for 60 mg vs. 200 mg; p = 0.0020 for 60 mg vs. 600 mg; p = 0.015 for 200 mg vs. 600 mg). The levels of HIF-1alpha, pimonidazole binding and alpha-tubulin acetylation were assessed by counting the percentage of immunohistochemically positive tumor cells and by grading the staining intensity. Grades (1, 2, 3) were multiplied with the percentage of cells in that grade, and the resulting parameters were added up over all grades. This sum was used as an estimation of the total amount of the respective antibody target. The sum was set to 1 for the vehicle control group. The table shows the relative decrease in the treatment groups, together with p-values of comparisons to the vehicle control. The table was created according to the data reported by reference [62].

400 mg bid to 3000 mg bid revealed no linear increase in plasma peak concentrations with the applied dose. The drug was taken up rapidly, but incompletely, and MTD was not reached. The achieved plasma concentrations were generally low for all dose levels tested. Inefficient intestinal uptake and/or rapid metabolism were assumed to be responsible for the low drug exposure and may have limited the antitumor efficacy. At least one woman with a clear cell carcinoma of the ovary experienced partial response. The toxicity observed was only moderate and did not exceed the grade 2 level in 12 out of 20 patients. Only one patient exhibited a severe allergic reaction (grade 4 angioedema), which was considered to be unrelated to 2-ME.

Similar results, i.e., safe long term application of 2-ME monotherapy, but low bioavailability and no objective responses, were reported in phase II trials of hormone-refractory prostate cancer [67] and relapsed or plateau-phase multiple myeloma [68]. The drug was safe, but exhibited suboptimal plasma levels, and was rapidly converted to the less active 2-methoxyestrone and inactivated by conjugation.

## 8. PRELIMINARY CLINICAL EXPERIENCES IN GLIOBLASTOMA MULTIFORME

A first phase II study has been performed in recurrent GBM to evaluate a continuous, long term 2-ME monotherapy [69]. The results showed that the drug was well tolerated. Sixteen patients were included in the study, 7 of whom were affected by their primary recurrence and 9 by their second recurrence. The drug was given orally 4 times per day at a dose of 1000 mg for the first 11 patients and then escalated to 1500 mg for the remaining individuals, thus reaching the highest doses of the earlier dose escalation trial with mixed solid tumors [66]. The primary endpoint was 6-months progression-free survival. Drug exposures, determined in pharmacokinetic studies, were too low and similar to those observed in the trial with mixed solid tumors at the same dose level. The rare grade 3 toxicities were transaminase elevation (3 cases) and hypophosphatemia (1 case). Six patients (37.5%) exhibited stable disease, including one minor response, suggesting modest anti-tumor activity of the drug in recurrent GBM.

These preliminary results led to a running study, that intends to elucidate the potential of a dual chemotherapy in recurrent GBM, combining the favoured alkylating drug TMZ and 2-ME. This trial is currently recruiting participants (FDA, NCT00481455). It is designed as a non-randomized, uncontrolled study with only a single treatment group, to explore safety and efficacy for this dual treatment. It will be interesting to observe, whether the results may justify a randomized comparison of this treatment versus TMZ alone.

However, the above cited clinical data regarding tumors outside the CNS and gliomas indicated that inadequate bioavailability is the major factor that limits the potential therapeutic benefits of 2-ME. Therefore, a major goal for the future should be the development of such 2-ME analogs, which reach higher and longer lasting plasma levels of the toxic molecule species. In the past decade, some efforts have been performed to synthesize large series of analogs and to test their biological activity *in vitro*. At the beginning, these efforts focused on enhanced cytotoxic properties, but recently, exciting developments were made in the field of reducing metabolic degradation and increasing plasma levels of the active species.

## 9. ANALOGES OF 2-METHOXYESTRADIOL

### 9.1. Compounds with Improved Cytotoxicity

Since 1995, Cushman and colleagues have synthesized various series of 2-ME analogs [70, 71, 72] carrying substituents in positions 2 and 6 in order to find compounds that inhibit the polymerization of bovine brain tubulin more efficiently and show enhanced cytotoxicity in the NCI screening panel of 55 tumor cell lines. In-

deed, some of the new molecules had properties similar to 2-ME, a few being even more potent. In general, the most efficient inhibitors of tubulin polymerization *in vitro* were the most cytotoxic compounds, although cytotoxicity was not necessarily accompanied by a significant tubulin inhibitor function.

A favourable substitution at the 2-position was the introduction of an unbranched chain with three non-hydrogen atoms, e.g. by replacing the methyl ether by an ethyl ether [70, 71], resulting in 2-ethoxyestradiol (Fig. 4b). This compound was much more cytotoxic in the whole panel of tumor cell lines in general (MGM value), as well as in the glioma cell line SF-539 (GI<sub>50</sub>). Both, MGM and GI<sub>50</sub> for SF-539, were reduced to about 5%, as compared with the parental drug. The IC<sub>50</sub> for inhibition of tubulin polymerization was 42% of the corresponding value for 2-ME [71]. Furthermore, molecules carrying an NOH moiety in the 6-position with either CH<sub>3</sub>CH<sub>2</sub>O or CF<sub>3</sub>CH<sub>2</sub>O in the 2-position (Fig. 4d) were also superior to 2-ME in all three parameters. A 2-ethoxyestradiol derivative with an additional ketone moiety in position 6 (Fig. 4c) was still highly potent. Later modifications at the 2-position by the same group [72] resulted in some other potent compounds, although not superior to 2-ME, such as 2-(E-3'-Hydroxy-1'-propenyl)estradiol and 2-(1'-Propynyl)estradiol (Fig. 4 e,f). Recently, Rao and colleagues synthesized series of ring-D-modified analogs [73, 74], some of which exhibited increased growth inhibition in HUVECs, MDA-MB-231 breast cancer cells, and U87MG glioma cells. The most potent compound resulted from the introduction of a delta<sup>14</sup>-bond in the D-ring [73]. These studies demonstrated that the potency of 2-ME may be further enhanced by modifications of the molecule that do not destroy its ability to interact with tubulin. However, the major problem to be solved for clinical applications, is the low bioavailability of the steroid compounds.

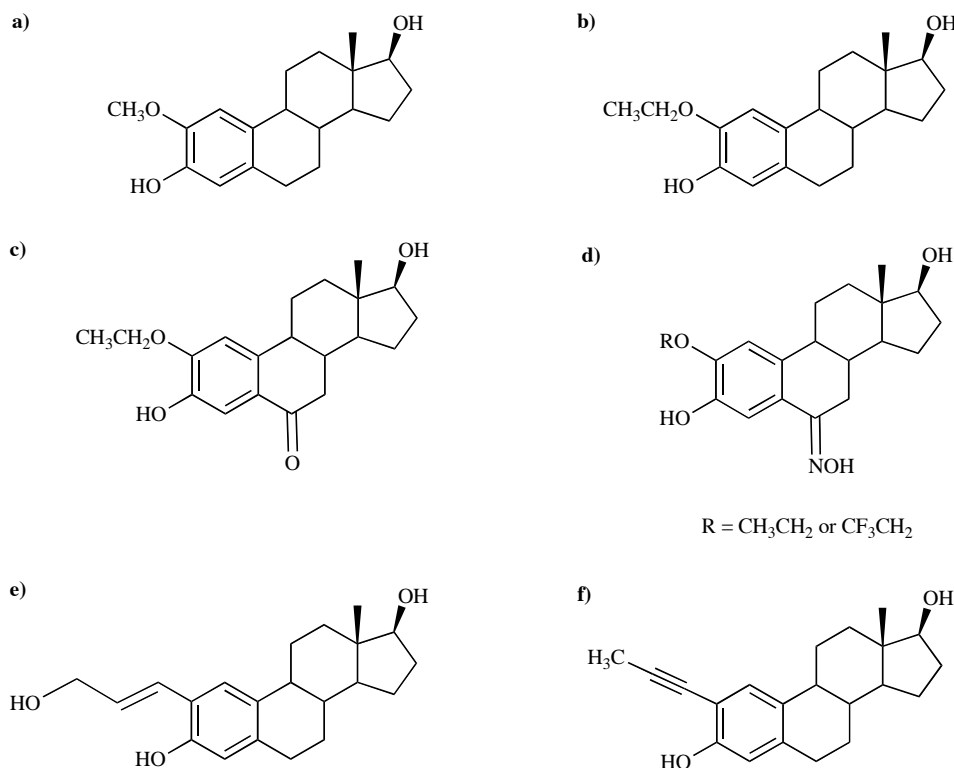
### 9.2. Inhibiting Metabolic Degradation and Enhancing Solubility

Especially oxidation of the hydroxyl group in position 17 of estradiol to estrone is believed to decrease *in vivo* efficacy because the estrone derivative has very limited activity. Moreover, conjugation of this moiety and of the hydroxyl group in position 3 to form glucuronide or sulphate, are major deactivation routes of 2-ME. In addition, demethylation of the 2-methyl ether could be detrimental.

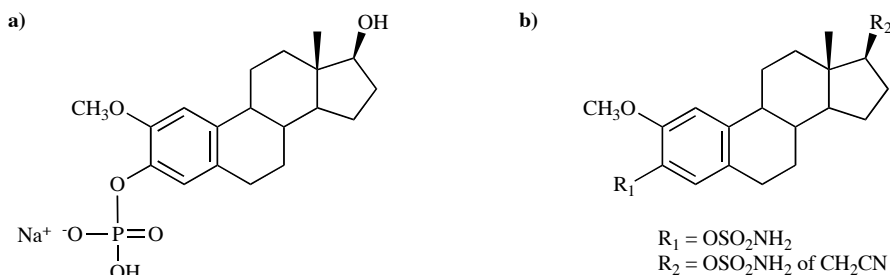
Therefore, another goal of Cushman's group was to create a series of analogs modified in positions 17 and 2, which could be expected to have a diminished potential for metabolic degradation, while possessing acceptable properties in the tubulin and cytotoxicity assays [75]. Four analogs fulfilled these conditions, namely 17 $\alpha$ -Methyl- $\beta$ -estradiol, 2-ethoxy-17 $\alpha$ -methylestradiol, 2-propynyl-17 $\alpha$ -methylestradiol, and 2-ethoxy-17-(1-methylene)estra-1,3,5(10)-triene-3-ol. The observed inactivity of many other analogs in the tubulin assay [75] could be explained by the hypothesis that steric bulks at both positions and branching of position 2 side chain were not well tolerated. However, as already suggested earlier [72], the inactivity of some compounds with ideal steric features suggested a role for electronic effects [75].

Since conjugation at position 17 is a major pathway of 2-ME detoxification, Agoston and colleagues investigated the question of whether substituents at the neighbouring position 16 may inhibit this process [76]. Larger substituents tended to result in lower cytotoxicity, but a few 16-modified compounds were comparable to 2-ME in assays with HUVEC and MDA-MD-231 breast cancer cells. *In vitro* assays revealed that glucuronidation of the hydroxyl group in position 17 was generally more important than sulfonylation. Comparison of 2-ME with all analogs in UDP-glucuronosyltransferase assays revealed rapid conjugation of all compounds tested, i.e., the aim of inhibiting this process by adding a substituent to the neighbouring position was not achieved.

Cushman's group also addressed the issue of low water solubility by a prodrug strategy [77]. The authors produced water soluble 2-ME derivatives by coupling phosphate to either the hydroxyl



**Fig. (4).** A series of potent analogs of 2-methoxyestradiol (a) with various small substituents at positions 2 and 6 were synthesized and tested by Cushman and colleagues [70-72]. Analogs (b) and (d) were actually more cytotoxic in a large panel of tumor cells, including gliomas, and were better inhibitors of tubulin polymerization. However, all these drugs did not address the major problem of low oral bioavailability.



**Fig. (5).** Improved bioavailability: The highly water soluble phosphorylated compound (a) was superior to 2-ME in tumor-bearing animals if applied via the intravenous route [77]. In animal models, the sulphamoylated compounds (b) were much more potent oral anti-tumor and oral anti-angiogenic drugs than 2-ME [78, 79].

group in positions 3, 17, or both. While the mean growth inhibition was low for the other two analogs, the 3-phosphate (Fig. 5a) exhibited at least similar properties as compared with the parental drug. The mean concentrations necessary for 50% growth inhibition in the whole NCI panel (55 cell lines), as well as in SF-539 glioma cells, were increased only 2-fold. As expected, in rats the 3-phosphate was metabolized rapidly (within one hour) to the active drug 2-ME. Unluckily, the oral application of the 3-phosphate did not enhance 2-ME plasma levels in rats relative to 2-ME feeding, demonstrating that this compound is not suitable as an orally applied prodrug. However, the enhanced water solubility facilitates intravenous application. Applied in this way, the 3-phosphate exhibited better responses in a Lewis mouse lung cancer model, as compared with 2-ME [77].

If  $\text{SO}_2\text{NO}_2$  is coupled to the hydroxyl groups instead of phosphate (Fig. 5b), these sulphamoylated compounds can no longer be metabolized *in vivo* to the parental drug 2-ME. A single oral dose of

10 mg/kg of the 3,17-O,O-bis-sulphamate did not yield any detectable amounts of 2-ME or its metabolites in rat plasma [78], demonstrating resistance to metabolism. However, sulphamoylation did not destroy the ability to bind the colchicin site of tubulin and conferred superior biological activity to the bis-sulphamate, which itself acts as the cytotoxic species. In contrast to 2-ME at the same dosage level, the compound was still detectable in rat plasma after 24 hours. Its bioavailability was around 85%. Most importantly, superior biological activity *in vivo* was found after oral application [78, 79]. While a daily dosage of 20 mg/kg 2-ME exhibited no significant effects in mouse xenograft models of MDA-MB-435 and MCF-7 cancer cells, the bis-sulphamate clearly inhibited tumor growth in both models. Similar results were obtained in the MCF-7 model [79] for an analog, in which the substituent in position 17 was cyanomethyl (Fig. 5b). The orally applied analogs were also superior to 2-ME in an assay testing the inhibition of blood vessel growth into bFGF-containing matrigel plugs, injected subcutane-

ously into the flanks of mice [79]. This demonstrated a superior ability to inhibit angiogenesis.

Taken together, the further development of improved compounds, such as sulphamoylated 2-ME analogs, to the level of clinical trials seems to be the most exciting perspective. At the preclinical stage, these compounds should be tested in an orthotopic rat glioma model, but using oral drug administration.

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