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The efficacy and toxicity of ATM inhibition in glioblastoma initiating cells-driven tumor models

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ABSTRACT

The Ataxia Telangiectasia Mutated (ATM)-mediated DNA damage response (DDR) is a major mechanism of resistance of glioblastoma (GB) - initiating cells (GICs) to radiotherapy. The closely related Ataxia Telangiectasia and Rad3-related protein (ATR) is also involved in tumor resistance to radio- and chemotherapy. It has been shown that pharmacological inhibition of ATM protein may overcome the DDR-mediated resistance in GICs and significantly radiosensitize GIC-driven GB. Albeit not essential for life as shown by the decade-long lifespan of AT patients, the ATM protein may be involved in a number of important functions other than the response to DNA damage. We discuss our current knowledge about the toxicity of pharmacologic inhibition of ATM and ATR proteins.

1. Background

Glioblastoma (GB - WHO, 2007; Grade IV; ORPHA n. 360) is a primary intracranial neoplasm that is almost invariably lethal, due to its infiltrating nature and resistance to therapy (Bush et al., 2017; Cihoric et al., 2017). Current therapies for GB are at best palliative: median survival is around one year and < 10% of patients is alive 5 years after diagnosis, Early symptoms may vary: from seizures to speech problems to sudden behavior changes; MRI investigation may then show a suggestive hyperintense lesion that rapidly develops if left untreated, with the tumor size in some cases multiplying by 32 in one month (Faguer et al., 2014). Standard treatment of GB is based on the Stupp protocol with some modifications introduced for individual trials. Temozolomide (TMZ) is the preferred chemotherapeutic agent in patients without prior exposure; lomustine is often used for tumors resistant to TMZ. A targeted therapy, bevacizumab, slows tumor growth but does not extend overall survival (Chinot et al., 2014; Gilbert et al., 2014). The biology of these tumors is such that curative doses of radio- and chemotherapy would be lethal to the surrounding brain. Given these limited treatment options at tumor recurrence, consideration for new therapies is a must.

Autopsy findings have shown infiltration of tumor cells even in the contralateral hemisphere suggesting that GB may be considered as a “whole brain” disorder. As a consequence, eradicating GB with currently available non-specific radio- and chemotherapy regimens would require treatment with elevated, toxic and probably lethal doses.

Cytotoxic T-lymphocyte protein 4 (CTLA4) and programmed cell death 1 (PD1)/programmed cell death ligand 1 (PDL1) have recently emerged as important biomarkers for immunotherapy of primary and metastatic brain cancer. Immunomodulatory agents targeting them separately or in combination have conferred significant clinical benefits to patients, albeit at cost of varying toxicity. Ongoing studies aimed to establish the specific composition of the brain tumors' microenvironment may lead to predict the potential activity of these immunomodulatory agents for brain cancer treatment (Passiglia et al., 2018).

To specifically target GB, we (Vecchio et al., 2014, 2015; Frosina et al., 2018b) and others (Biddlestone-Thorpe et al., 2013; Karlin et al., 2018; Durant et al., 2018) have recently identified new radiotherapeutic procedures. The novel radiotherapy regimens are based on the capacity of ATM inhibitors such as KU60019, specifically to stimulate proliferation of quiescent GB-Initiating Cells (GIC). This is based on the concept that the ineffectiveness of radiotherapy is due to surviving quiescent tumor initiating cells that are thought to be the root cause of different neoplastic disorders and resistance. ATM inhibitors appear to induce proliferation in residual, resistant GICs, thus making them sensitive to radiotherapy (Vecchio and Frosina, 2016).

In view of their clinical employment, thorough safety evaluation of

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ATM inhibitors has to be performed. We review here our current knowledge about the safety of ATM inhibitors as obtained in preclinical studies. We also discuss the potential side effects of pharmacological ATM inhibition as inferred by the pleiotropic consequences of ATM genetic deficiency.

2. Pleiotropic effects of genetic ATM deficiency

Mutations in the ATM gene located on the long arm of chromosome 11 (11p22-23) cause a rare autosomal recessive disorder, ataxia telangiectasia (AT) characterized by neurodegeneration with progressive cerebellar ataxia, oculocutaneous telangiectasias, hypersensitivity to ionized radiation with elevated incidence of lymphomas, defects in the immune response with recurrent pulmonary infections, diabetes, premature aging and infertility (Stagni et al., 2018). Epidemiological studies have shown that AT heterozygotes as well may be susceptible to ionizing irradiation and have a higher risk of cancers and metabolic disorders than wild type individuals (Zaki-Dizaji et al., 2018). Unrepaired DNA double-strand breaks (DSBs) and defects in the signal transduction pathway activated by DNA damage [DNA damage response (DDR)] can be observed at the molecular level in AT patients. ATM senses radiotherapy and chemotherapy-induced DSBs and signals to cell cycle checkpoints and the DNA repair machinery preventing the entry of cells with damaged or incompletely replicated DNA into mitosis. This regulation may be particularly evident in cells with a defective G1 checkpoint, a common feature of cancer cells harboring p53 mutations (Qiu et al., 2018). Through cell cycle analyses, it has been demonstrated that low-dose irradiation preferentially activates the ATM pathway and inhibits cell growth and arrests the cell cycle in p53-mutant rather than p53 wild type cells. Consistently, the ATM pathway is elicited with higher efficiency by radiation after wild type cells loose p53 (Li et al., 2018).

Despite our improved knowledge of ATM function at the molecular level, several clinical aspects of the AT disorder still remain unexplained. For instance, an unexpected favorable course of AT symptoms in one patient with initial typical phenotype has been described. A female child with a compound ATM genotype consisting of an in-frame deletion and a missense mutation causing undetectable ATM protein activity, developed by the age of 3 years all the typical symptoms of the AT disorder. However, 9 years later the AT symptoms gradually mitigated in the absence of any innovative treatment and the only residual neurologic symptom consisted in a mild choreic disorder. As a possible molecular explanation for this unusual disease regression, transcriptome analyses showed that whereas 90% of RNAs were expressed as in classic AT patients, the remaining 10% were as in healthy controls, indicating that endogenous modifying factors may exist that mitigate the AT phenotype (Leuzzi et al., 2018). ATM is certainly an important factor in a number of physiological functions as the multiple consequences of its impaired activity demonstrate.

2.1. Cardiovascular and respiratory systems

ATM may be involved in control of atherosclerosis and cardiovascular disorders. Irradiated bovine aortic endothelial cells activate and upregulate ATM that causes increased transcription of endothelial nitric oxide synthase (eNOS), a generator of nitric oxide (NO) (Nagane et al., 2018). In turn, NO inhibits cell death, as well as causes cellular senescence. Hence, radiation-induced DDR uses ATM kinase to upregulate eNOS transcription and NO generation, leading to cellular senescence, which may play a critical role in radiation-mediated cardiovascular injury. A relationship between ATM and cardiovascular disorders might be suggested by the frequent presence in patients with coronary artery disease (CAD) of the single nucleotide polymorphism [(SNPs); rs189037C > T] in the promoter sequence of the ATM gene. In a case-control study performed in Chinese Han populations, 652 CAD patients diagnosed by coronary angiography and 656 unaffected control subjects were enrolled. The presence of the SNP was much higher in patients than in control subjects. The risk linked to the presence of the SNP was especially evident in males and smokers (Ding et al., 2018). ATM deficiency may result in defective autophagy in the heart during early myocardial infarction (MI). MI with significantly reduced ejection fraction was induced in wild type and ATM heterozygous knockout mice by ligation of the left anterior descending artery. ATM heterozygosis was associated to delayed autophagy during MI as determined by reduced LC3-phosphatidylethanolamine conjugate (LC3-II) and cathepsin D protein levels and augmented p62 and aggresome accumulation. Consistently, inhibition of ATM by the specific inhibitor KU55933 resulted in defective autophagy in cardiac cells (Thrasher et al., 2018).

During cerebral ischemia, glucose 6-phosphate dehydrogenase (G6PD) activity in pentose phosphate pathway (PPP) is activated via heat shock protein 27 (HSP27) -phosphorylation by ATM kinase (Yamamoto et al., 2018). This mechanism might serve as an endogenous antioxidative system and its importance on the metabolism of rat cortex was investigated using middle cerebral artery occlusion (MCAO). The intracerebroventricular injection of ATM kinase inhibitor KU55933 significantly reduced HSP27 phosphorylation and G6PD activity after MCAO and resulted in increased infarct size (Yamamoto et al., 2018).

Altered redox balance in alveolar epithelia may underlie respiratory deficiency in AT, since ATM protein plays a protective role from lung inflammation and fibrosis driven by oxidation damage. To investigate the bronco-alveolar sensitivity to oxidatively damaged DNA, experimental damage was induced in ATM-deficient mice using the radiomimetic drug bleomycin (BLM) (Duecker et al., 2018). BLM or saline were instilled through the oropharyngeal route into the lungs of ATM-mutant and wild-type mice. BLM administration resulted in marked lung inflammation and fibrotic damage in ATM-deficient mice with consequent respiratory loss of function. As a radiomimetic drug, bleomycin induced DNA damage that effectively triggers ATM activation, including DSB and oxidized lesions. Consistently, ATM deficiency resulted in increased cell death, delayed resolution of gamma-H2 AX expression and elevated levels of ROS in pulmonary epithelial cells treated with BLM (Duecker et al., 2018).

2.2. Central nervous and immunological systems

Whether the neurological aspects of AT are a direct consequence of defective DDR is unclear. It has been proposed that the neurodegenerative process in AT might be linked to the defective cytoplasmic roles of ATM protein. In non-cycling cells such as neurons, ATM is mostly found in the cytoplasm. In particular, ATM co-localizes with organelles such as mitochondria or peroxisomes that produce ROS which have been implicated in neurodegenerative processes. Further, ATM is associated with synaptic vesicles and a role for ATM in regulating cellular homeostasis and autophagy has been suggested (Choy and Watters, 2018). Accordingly, in ATM-deficient neurons, signaling by vesicle release is reduced and it has been shown that ATM binds to both clathrin and components of the AP-2 complex, indicating a possible role of the multifunctional ATM protein in endocytosis and recycling of vesicles as well (Cheng et al., 2018). Super-resolution microscopy and co-immunoprecipitation studies have shown that ATM associates with excitatory [VGLUT1(+)] vesicles only. Since deficiency of ATM is often accompanied by increased levels of ATR and vice versa, it has been hypothesized that ATM and ATR proteins may take part to the mechanisms that control the inhibitory/excitatory balance in the nervous system (Cheng et al., 2018).

Class switch recombination (CSR) of Ig heavy chains (IgH) in B lymphocytes switches IgH constant regions to change antibody functions (Panchakshari et al., 2018). Formation of DSBs in donor and acceptor switch regions is the initiating steps of CSR. This finely tuned phenomenon is completed by fusion of the donor and acceptor regions

via non homologous end joining or, alternatively, using junction microhomologies (MHs). Defective DSB repair possibly linked to defective ATM function may impair CSR end-joining and cause increased MH utilization (Panchakshari et al., 2018).

2.3. Infection

The human herpes virus Epstein-Barr (EBV) is a causative factor for both epithelial and lymphoid malignancies. For instance, the epithelial nasopharyngeal cancer (NPC) is often associated with latent EBV infection in Southern China and Southeast Asia populations (Lung et al., 2018). Reactivating the lytic cycle of EBV is being explored as a therapeutic strategy in such cancers. Rapamycin can enhance the viral reactivation triggered by the EBV lytic inducer sodium butyrate by upregulating the expression of EBV lytic proteins. In particular, the EBV immediate-early lytic promoters were activated after rapamycin treatment. On the contrary, inhibition of the ATM-mediated DDR pathway by siRNA targeting the ATM gene expression greatly reduced the virus reactivation induced by rapamycin. Hence, the ATM-mediated DDR is probably involved in the process of EBV reactivation by rapamycin (Wang et al., 2018). Consistently, miR-BARTs, the EBV-encoded miRNAs derived from BamH1-A rightward transcripts, have been found expressed at elevated levels in NPC and demonstrated to target various cellular and viral genes implicated in nasopharyngeal carcinogenesis including ATM. Some BARTs miRs may negatively regulate the expression of ATM, by binding to multiple sites on its 3′-UTR and accordingly, reduced expression of ATM is a common finding in NPC. The EBV miRNAs may work co-operatively to downregulate ATM activity thus inhibiting EBV reactivation and maintaining virus latency and tumorigenicity (Lung et al., 2018).

Human papillomaviruses (HPVs) are the etiologic agents of cervical cancer and are linked to the development of many other anogenital and oropharyngeal cancers. Similar to EBV, replication of high-risk HPVs requires the activation of the ATM and ATR DNA repair pathways. HPVs activate these pathways in response to DNA breaks induced by viral proteins involved in virus integration and independently of other exogenous factors. Most of those DNA breaks occur in cellular rather than viral genomes (Mehta and Laimins, 2018).

The activation of ATM is dampened in T cells from subjects with hepatitis C virus (HCV) infection (Zhao et al., 2018). The ATM activation was also diminished in healthy T cells exposed to ATM inhibitor or to HCV (core protein) that inhibits the phosphoinositide 3 kinase pathway, mimicking the biological effects in HCV T cells. Importantly, the ectopic expression of ATM was sufficient to cope for the reduced ATM activity and cell dysfunctions in HCV T cells. Insufficient ATM activity may lead to increased DNA damage and apoptotic death in HCV-infected T cells 5915 (Zhao et al., 2018).

Development of gastric cancer is often associated to Helicobacter pylori infection. The mechanism of gastric carcinogenesis induced by *H. pylori* infection includes the production of DNA damage and epigenetic dysregulation. In particular, DSBs triggering the ATM-mediated DDR increase in gastric epithelial cells and in biopsy specimens from patients with gastric cancer after *H. pylori* infection. The ATM activation is further associated to epigenetic changes such as histone H3 and H4 hyperacetylation and DNA promoter hypomethylation (Santos et al., 2018).

2.4. Tumor resistance

Through the study of specimens from a large cohort of human breast cancer subjects, it has been shown that patients with tumors that expressed high levels of zinc finger E-box binding homeobox 1 (ZEB1) responded poorly to chemotherapy (Zhang et al., 2018b; Rozpedek et al., 2018). The ZEB1 protein has been implicated in resistance to chemotherapeutic drugs such as epirubicin (EPI) in breast cancer cells. The suggested mechanistic basis is transcriptional activation of ATM protein kinase leading to improved homologous recombination (HR)-mediated DNA damage repair of EPI-induced DSBs. Increased resistance to EPI after the ectopic expression of ZEB1 has been demonstrated in vivo as well, using an immunodeficient mouse xenograft model (Zhang et al., 2018b).

Prolactin (PRL) may improve survival of breast cancer cells treated with doxorubicin or etoposide. Two different HSP90 inhibitors counteracted this effect of PRL and caused a decrease in levels of both native and phosphorylated ATM protein. Consistent with an involvement of ATM in this context, the specific ATM inhibitor KU55933 abolished the PRL-mediated improvement of survival in treated cells. Similar inhibitory effects on the PRL pro-survival activity were observed after administration of short interfering RNA directed against ATM. ATM protein may thus contribute to the PRL-JAK2-STAT5-HSP90 pathway in causing resistance of cancer cells to chemotherapy (Karayazi Atici et al., 2018).

Microglia has been considered as a radioresistant cell type. The putative radio-resistance of microglia was studied independently of its physiological brain environment after X-irradiation. A significantly higher survival rate of isolated microglia accompanied by a decreased proliferation rate was found after X-irradiation as compared to different cell types. No variation of antiapoptotic pathways was detected but ATM was significantly upregulated suggesting that alterations of microglia that resist irradiation may include upregulation of ATM and the DDR (Menzel et al., 2018).

The epithelial mesenchymal transition (EMT) is involved in tumor progression with metastatic expansion as well as the generation of tumor cells with stem cell properties that play a major role in resistance to cancer treatment (Roche, 2018). Androgen independent prostate cancer [also called castration resistant prostate cancer (CRPC)] is highly lethal and characterized by widespread EMT (Montanari et al., 2017). The expression levels of ATM kinase have been found increased in CRPC tissues as compared to hormone-dependent prostate tumor tissues. CRPC cell lines have been established and ATM knockout cells have been obtained via lentivirus infection. The delete EMTdelete and migration capacity of these ATM-deficient cells were significantly reduced as compared to ATM wild type cells, suggesting that ATM might be involved in EMT and migration of CRPC cells. Antibodies against delete PDL1delete and inhibitors of JAK 1 significantly reduced cell migration ability as well as the expression of EMT associated markers. Hence, EMT, metastasis and progression of CRPC might be counteracted through inhibition of the ATM-JAK-PDL1 signaling pathway (Zhang et al., 2018a).

The multifunctional cytokine, interleukin-6 (IL-6), stimulates the growth of GB cells but not of normal neural progenitor cells. Further, IL-6 has been shown to promote radioresistance in GB cells (Lim et al., 2018). Ablation of ATM eliminates the radioresistance effect of IL6 and re-sensitizes GB to radiation DNA damage. Hence, targeting the ATM-mediated DDR might be a potential approach to revert the IL-6-promoted radioresistance in GB (Lim et al., 2018).

Mantle cell lymphoma (MCL) is a highly chemo-refractory tumor with generally poor outcome. ATM is one of the most frequently mutated genes in MCL and recent experimental evidence has demonstrated that this mutational status can be taken advantage of using radiotherapy. Under conditions of ATM deficiency, radiotherapy may represent a viable treatment option for mantle cell lymphoma (Ahmed et al., 2016).

3. GB radiosensitization via ATM inhibition

Recurrence in GB has been linked to specific cell populations (GICs) refractory to radio- and chemotherapy due to their quiescent state, from which they exit to regenerate the tumor once therapy has ceased (Bao et al., 2006). Their quiescence is not due to prolonged G1 but involves the establishment of a G0 phase. Highly resistant GICs can be found close to necrotic tissues, in specific niches characterized by an hypoxic

and acidic microenvironment. Transcriptional profiling reveals that these cells down-regulate genes associated with cell-cycle progression and upregulate genes classified as tumour suppressors (Aulestia et al., 2018). In particular, the DDR may be constitutively activated and further elicited by therapeutic treatments (Rozpedek et al., 2018; Frosina, 2009).

RT induces mainly DSB lesions on DNA, while TMZ, a cytotoxic imidazotetrazine routinely included in the therapeutic schedule, forms O^6-methylguanine, which mismatches with thymine in subsequent DNA replication cycles. Thus, both RT and TMZ treatments typically trigger the DDR. In this context, the DDR-mediated activation of the G2/M checkpoint acts as a quiescence-inducing, prosurvival mechanism that gives time to the cells to repair their DNA. Increasing the RT dose per fraction such as in hypofractionated stereotactic radiotherapy may actually be counterproductive by enhancing the ATM-mediated DDR resistance mechanism with little benefit if any to patients' survival (Scoccianti et al., 2018). On the contrary, by flushing GICs out of the G2/M checkpoint, ATM inhibiting drugs may specifically induce their proliferation thus significantly improving tumor response to RT that is toxic to proliferating cells only.

Radiosensitivity of GB cells may be enhanced by ATM gene silencing as well, Expression levels of the ATM gene and protein were down-regulated in the established glioma cell line U251 by transfection with an siRNA-ATMpuro lentivirus. Colony formation assays showed lower cell proliferation and survival in irradiated ATM-depleted cells as compared to irradiated control cells. The level of double-stranded DNA breaks, the rate of cell apoptosis, the number of cells in the G2 phase and the activity of pro-apoptotic caspase-3, caspase-8 and caspase-9 were all increased in ATM-silenced as compared to control cells. In vivo analysis in mouse models created by implantation of the transfected cell lines showed that the amount of necrosis and hemorrhage was higher in silenced with respect to control tumors (Li et al., 2016).

We (Vecchio et al., 2014, 2015; Frosina et al., 2018b) and others (Biddlestone-Thorpe et al., 2013; Karlin et al., 2018; Durant et al., 2018) have demonstrated that repeated treatments with ATM inhibitors followed by IR, substantially enhanced the toxic effect of RT in vitro and even eradicated radio-resistant cells, whereas GICs treated with vehicle plus radiation recovered early and expanded. In vivo, the orthotopic tumor response to RT was significantly increased in the presence of ATM inhibitors and this effect was more rapid in tumors displaying reduced expression of tumor suppressor p53 and elevated expression of anti-apoptotic PI3K (Vecchio et al., 2014; Biddlestone-Thorpe et al., 2013). ATM inhibitor molecules may be remarkably stable: no significant degradation of KU60019 was observed up to 168 h incubation at body temperature (37 °C) (Vecchio et al., 2015). The pharmacokinetics and pharmacodynamics of ATM inhibitors have been recently improved. While earlier ATM inhibitors such as KU60019 were essentially unable to cross the blood brain barrier (BBB) and had to be directly (and invasively) delivered intracranial, last generation inhibitors such as AZ32 and AZD1390 have been specifically designed for and effectively cross the BBB (Karlin et al., 2018; Durant et al., 2018). Similar to tumors from adult patients, pediatric GICs can be radiosensitized after exposure to ATM inhibitors (Vecchio et al., 2015; Frosina et al., 2018b). Taken together, these findings suggest that ATM inhibition may permit radiosensitization of a wide range of adult and pediatric HGGs.

4. Toxicity of pharmacologic ATM inhibition

Median survival in two large cohorts of AT patients, one prospective and one retrospective, was 25 and 19 years respectively (Crawford et al., 2006). Hence, ablation of ATM function is compatible with life. Accordingly, acute toxicity of ATM inhibitors, both as small molecules or small interfering RNA is limited (Li et al., 2016).

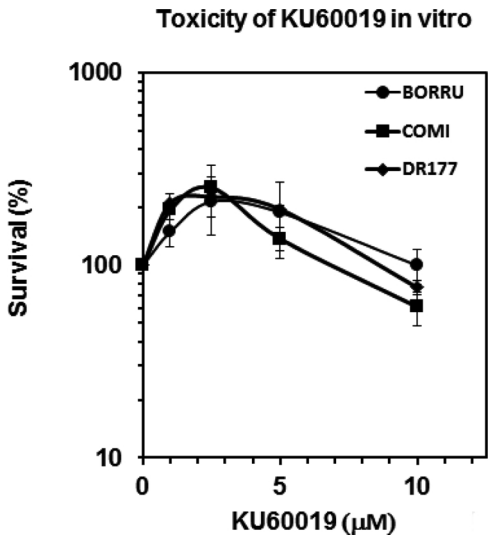

Fig. 1. In vitro toxicity of KU60019 on GICs. Metabolic activity of different GICs in the presence of increasing concentrations of KU60019. Data pooled from this study and ref. (Vecchio et al., 2014), with permission.

4.1. In vitro studies

In vitro studies with KU55933 that inhibits ATM protein with IC_{50} = 13 nM and effectively radiosensitizes human tumor cells at 10 μM showed no significant reduction of clonogenic survival at this concentration (Hickson et al., 2004). The low toxicity of KU55933 was later confirmed and extended to a number of additional cell types including glioma initiating cells (GICs) (Raso et al., 2012) and human corneal epithelial cells (Alekseev et al., 2014).

The improved ATM inhibitor KU60019 (IC_{50} = 6.2 nM) similarly displays no significant toxicity up to 10 μM while being effective at 1 μM as a radiosensitizing agent towards a wide range of tumor cell lines including primary GICs (Vecchio et al., 2014; Raso et al., 2012) (Fig. 1). Stimulation of cell growth and clonogenic survival was in fact observed in the range 1–5 μM (Fig. 1).

Conventional cytogenetic analyses have shown that treatment with KU60019 plus radiation does not increase chromosomal aberrations in COMI and DR177 GICs as compared to vehicle plus radiation, indicating that radiosensitization by ATM inhibitors is not clastogenic.

We further determined if KU60019 could increase the point mutagenicity of IR using the ouabain-resistance mutation assay. The mechanistic basis of this assay relies on the lethal effect of binding of ouabain to the Na^+/K^+-dependent ATPase enzyme which is involved in sodium and potassium transport across cell membranes and is essential for life. Mutations that result in limited structural changes of the ATPase enzyme decrease the affinity for ouabain and produce viable ouabain-resistant mutants (Clark and Li, 1986). GICs were exposed to KU60019 plus radiation and a period of 15 days was allowed for the phenotypic expression of mutations at the Na^+/K^+-dependent ATPase locus. No significant increase of mutation frequency in radiosensitized cells as compared to vehicle plus radiation-treated cells was recorded, indicating that the radiosensitization through ATM inhibition does not increase point mutations.

Similar to KU60019, the improved KU59403 ATM inhibitor was an effective chemosensitizer at a concentration of 1 μM which showed no inherent cytotoxicity in vitro (Raso et al., 2012; Batey et al., 2013).

4.2. In vivo studies

Unlike orthotopic tumors driven by established cell lines such as U87 and U1242 which are poorly infiltrating and relatively contained, tumors driven by primary GICs may closely mimic the clinical tumors of

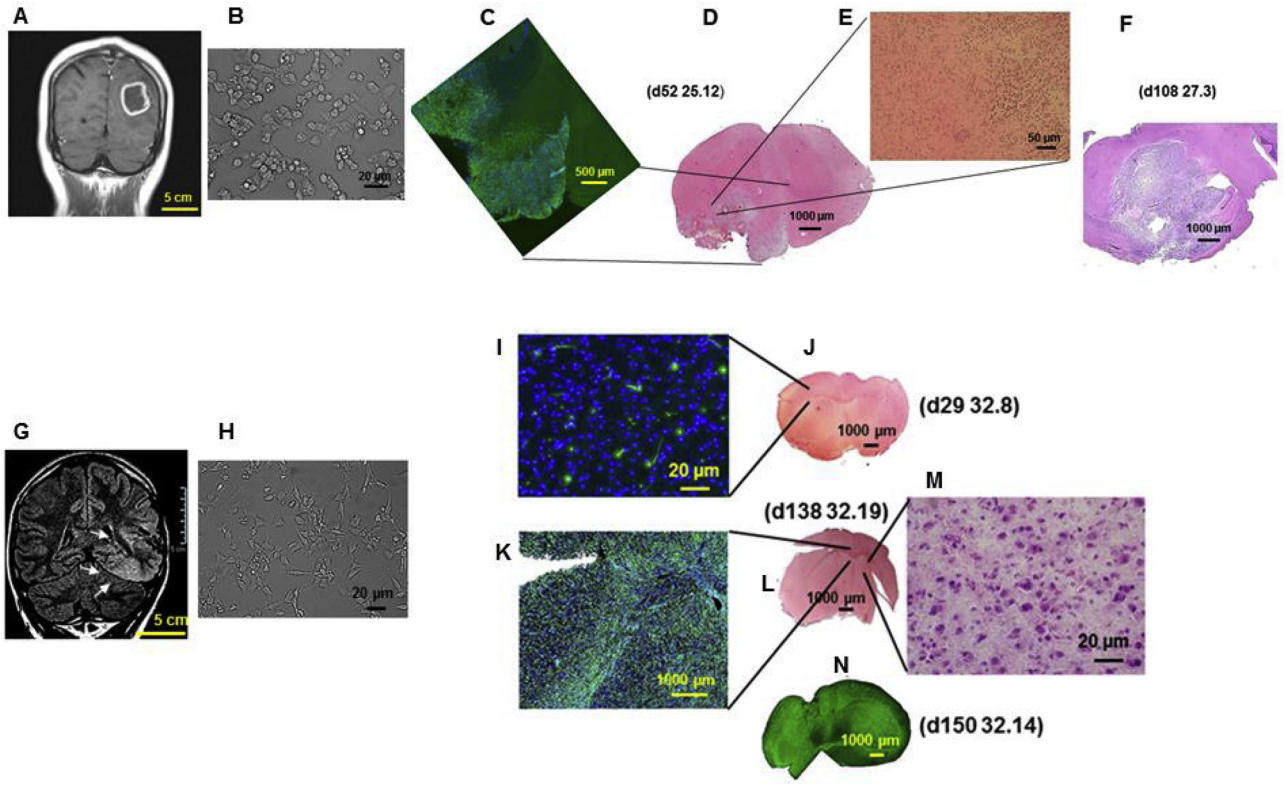

Fig. 2. Faithful animal modeling of human gliomas using GICs. A–F. Adult COMI GB. A. Coronal T1 MRI showing a left parietal-occipital HGG compact lesion with irregular walls, heterogeneous contrast and necrotic core. B. GICs isolated from the surgically-removed lesion and cultured under serum-free conditions. The cells layered in matrigel-coated flasks growing slowly (population doubling time: 60 h) and maintaining indefinite proliferation capacity (Vecchio et al., 2014). C–F. Following injection of GICs in the left corpus striatum of immunodeficient mice, the developing orthotopic tumor was visible after H/E staining (D,F) with a growth pattern similar to the patient's lesion: a compact tumor mass with irregular walls (E) exerting significant mass effect on the adjacent structures (F). Immunostaining at d52 showed that most of the tumor was positive for the stem cell marker nestin (C), indicating its stem cell predominant composition. Mouse ID numbers 25.12 and 27.3 are indicated for reference purposes with the days (d) of tumor development. G–N. Pediatric 239/12 anaplastic astrocytoma. G. Unlike adult COMI, coronal FLAIR MRI at progression shows a highly infiltrating tumor with little compressing attitude. H. Isolated GICs layering and slowly proliferating in matrigel-coated flasks. One month (d29 – mouse 32.8) after GICs injection in the left corpus striatum (J), scattered GICs could be detected in the normal brain parenchyma after immunostaining with anti-nestin antibody (I) but not after H/E staining (*J*). Only after more than four months of orthotopic tumor growing, (d138, mouse 32.19; d150 mouse 32.14) a highly infiltrating tumor reminiscent of its clinical counterpart was observable after both H/E staining (L,M) and immunostaining for nestin (K,N). Modified from ref. (Frosina et al., 2018b) with permission.

origin (Frosina, 2013). In this regard, the different growth patterns of the orthotopic adult glioblastoma COMI (acronym of patient's name) and the pediatric anaplastic astrocytoma 239/12 are taken here as suitable examples (Fig. 2).

The COMI tumor was surgically removed from a 48 years old male patient whose early symptoms were two episodes of violent headache in the left parietal-occipital region. In the following days, the patient's conditions worsened with increased space-time disorientation. He was therefore subjected to brain MRI which showed a voluminous expansive lesion in the left parietal-occipital region with compression of adjacent structures that was in the first instance compatible with an high grade glioma (HGG) lesion (Fig. 2A). After surgery, histological analysis of the lesion led to diagnosis of GB. Despite postoperative chemo- and radiotherapy, the patient died 413 days after diagnosis. Tissue surplus to diagnostic requirements was used immediately after surgery for isolation of GICs according to (Raso et al., 2012). In matrigel-coated flasks and under serum–free culture conditions, the cells maintained proliferation capacity and expanded (Fig. 2B). After injection in the left corpus striatum of immunodeficient NOD SCID mice, a progressively expanding lesion developed with growth characteristics similar to those of the clinical tumor (Fig. 2D–F). Immunostaining revealed that most of the tumor was positive for the human stem cell marker nestin indicating its stem cell predominant component (Fig. 2C).

The 239/12 tumor was operated in a 14 years old boy who presented to the Emergency Department of the Giannina Gaslini Children's Hospital in Genova, Italy for seizure attacks. MRI showed a highly infiltrating lesion with limited compression of surrounding tissues (Fig. 2G).

The lesion was partially removed surgically and histological analysis led to diagnosis of anaplastic astrocytoma (WHO grade III). The patient was subjected to postoperative chemotherapy with TMZ and intensity modulated radiotherapy (IMRT) which could not avoid his death 551 days after diagnosis. GICs were isolated and cultured as above (Fig. 2H) and once injected intracranial in NOD-SCID mice, led to development of orthotopic tumors faithfully reproducing the growth pattern of the clinical tumor of origin: the orthopic tumor grew slowly and was highly infiltrating with limited compressive attitude. Immunostaining with nestin antibody could reveal scattered tumor cells in the normal brain parenchyma (Fig. 2I) which gradually infiltrated the whole brain (Fig. 2K–N). Due to the absence of evident tumor masses, hematoxylin/eosin staining could reveal the presence of the infiltrated tumor only at late times of development (d138 – Fig. 2M). Hence the different growth patterns of COMI and 239/12 clinical tumors were faithfully reproduced in the mouse brain implanted orthotopically with the in vitro cultured primary GICs. This is different from the orthotopic tumors implanted orthotopically using established cell lines isolated many years ago and highly selected in vitro such as e.g. the U87 cell line, whose developed tumors are often contained thus representing a more direct and accessible target to experimental drugs. Further, the DNA profile of currently used U87 has been recently found different

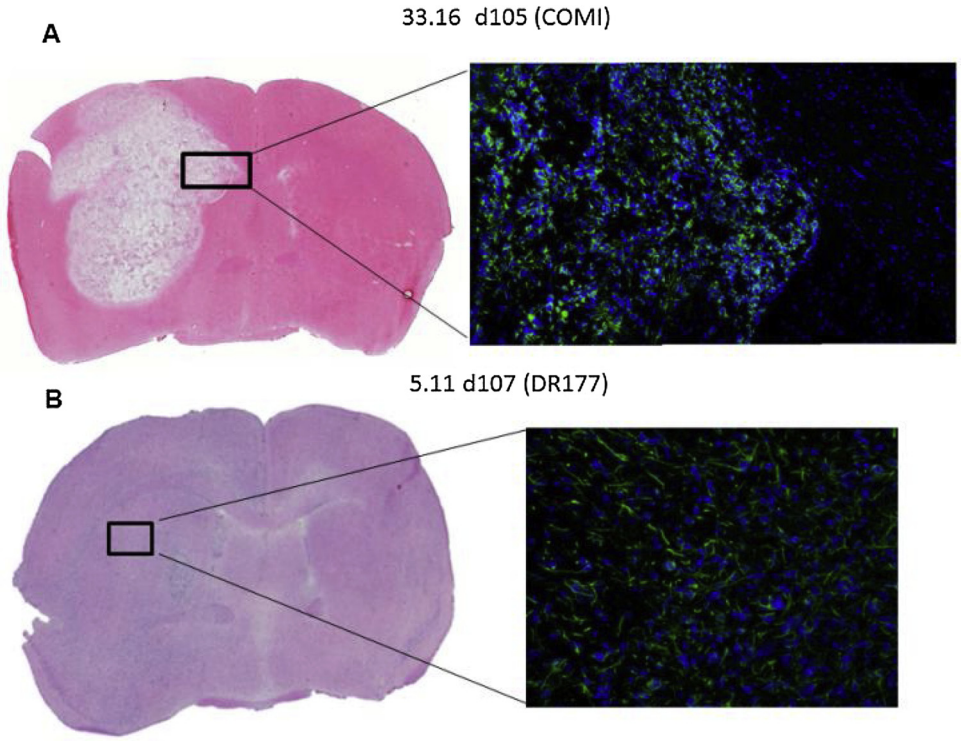

Fig. 3. The multiform nature of orthotopic GIC-driven adult GBs. A. Further showing of COMI growth properties. GICs were isolated from the adult GB COMI, expanded and injected in the left corpus striatum of immunodeficient NOD SCID mice. 105 days later a voluminous expansive lesion exerting elevated compressing effect on adjacent structures was observed after H/E staining of tissue sections (left). Immunostaining showed that most of the tumor expressed the stem cell marker nestin (green spots). Nuclei stained with DAPI appear in blue. The mouse ID number 33.16 is indicated for reference purpose. B. GICs were isolated from the adult GB DR177 and injected orthotopically in immunodeficient NOD SCID mice as in (A). 107 days later a highly infiltrating orthotopic tumor with limited compressive attitude over adjacent structures was barely detectable after H/E staining but evident after immunostaining for the stem cell marker nestin (green spots). All nuclei were stained with DAPI in blue. The mouse ID number 5.11 is indicated for reference purpose. Data pooled from this study and ref. (Vecchio et al., 2014), with permission (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

from that in the tumor of origin, indicating a possible contamination with a different cell line (Allen et al., 2016). Translational research on new effective therapies for GB could be greatly improved by using multiple animal models of human GB driven by primary GICs rather than established cell lines.

Initial in vivo studies performed using orthotopic tumors driven by established cell lines (U87 and U1242) showed no gross abnormalities in normal mouse brain after locoregional treatment with KU60019 and radiation (Biddlestone-Thorpe et al., 2013). The in vivo toxicity of KU60019 was later investigated in detail in both tumor-free and tumor-bearing animals. Since KU60019 cannot cross the BBB (Vecchio et al., 2015; Frosina et al., 2018b), it had to be administered directly into the brain using three different procedures. In the first one, the drug was injected by a Hamilton syringe stereotactically positioned under the same coordinates used for injections of GICs (Fig. 3) (Vecchio et al., 2014).

Tracking with methylene blue showed that the KU60019/dye mixture diffused in the whole brain depth down to the tumor lesion as identified via MRI. 500 nmoles of KU60019 i.c. injected by a stereotactically-placed syringe caused no evident neurological symptoms. Histological analysis of brains revealed no alterations of normal brain tissues 1 day or 14 days after injection as compared to vehicle (Vecchio et al., 2014). In the second and third delivery protocols, the toxicity of prolonged KU60019 exposures was investigated (Vecchio et al., 2014).

In the second one, 840 nmoles of KU60019 were i.c. delivered using AlzetVR mini-osmotic pumps at a rate of 120 nmoles/day over 7 days. At the fifth day of pump activity, the tracking mixture of KU60019 and methylene blue had remained predominantly located in the upper portion of the brain thus poorly involving the tumor tissue below. Delivery by osmotic pumps may thus permit only limited drug diffusion to the brain, possibly due to insufficient liquid flow and positive pressure exerted by the pump itself. No evident behavioral changes were observed in KU60019.treated mice during or at the end of the procedure (day 7—pump removal). Similarly, no evident symptoms were observed 10 days later and no macroscopical alterations or tissue damages were observed in eight organs of the mice examined by histopathology (Vecchio et al., 2014).

In the third procedure, 1000 nmoles of KU60019 were dissolved in both components 1 (fibrinogen/calf aprotinin) and 2 (thrombin) of TisseelVR (Baxter). The two KU60019-containing components were mixed and immediately applied to the brain surface of six anesthetized mice where they formed a drug-trapping clot. Thereafter, the drug-containing clot was reabsorbed at variable rates with complete reabsorption occurring 18 days after application. No symptoms were observed in the KU60019-treated mice as compared to control mice treated with vehicle (ethanol) or saline. No histological difference was observed in eight organs of the KU60019-treated mice with respect to those of control mice (Vecchio et al., 2014).

We then investigated whether the association of KU60019 with radiation could damage normal tissues. Tumor-free mice were subjected to convection enhanced delivery (CED) with 3 nmoles of KU60019 followed by three IR fractions of 2.5 Gy each, delivered 3,4 and 5 days later. Control mice were treated with vehicle only and irradiated. Radiation was delivered to the head of anesthetized mice by a Leipzig applicator of 10-mm diameter endowed with a ^{192}Ir source. The delivered dose was measured by two radiochromic films placed over the head and under the snout respectively and the dose distribution profile was determined. The median dose delivered to the whole brain was 60% of the prescription dose (2.5 Gy) and the mean dose rate was 1.7 Gy/min (range 1.3–2.7). Histopathological analyses detected no difference between brain tissues treated with KU60019 plus IR and brain tissues treated with vehicle plus IR at either 10 or 49 days after the end of treatment. Similarly, no significant difference was observed 21 days after the end of treatment between mice treated with KU60019 + IR and mice treated with vehicle + IR in bone marrow, heart, kidneys, liver, lungs, spleen and testes. Taken together, these data indicate that one CED with 3 nmoles of KU60019 followed by radiotherapy does not cause evident neurological consequences nor damages normal tissues more than radiotherapy alone.

We have recently observed that multiple KU60019 administrations may yet cause neurological symptoms. Orthotopic high grade gliomas were induced in immunodeficient mice by intracranial injection of adult COMI GICs (Fig. 2A-F, A keep the 3: Fig. 3A further shows an example of COMI GIC-driven tumor). Fifty two days after GICs injection

in the left brain hemisphere, mice were subjected to CED with 5 nmoles of KU60019 or vehicle (2.5% ethanol in 0.9% NaCl) followed by irradiation with 0.5 Gy at d56, 57 and 58. This radiosensitization cycle was then replicated with CED at d59 followed by 1.5 Gy at d63 and with CED at d66 followed by 1.5 Gy at d70, 71 and 72 (total dose delivered: 7.5 Gy). At this time (d71, d72), significant hyperkinesis and lateralization of ambulation (ring walking) were observed in KU60019-treated mice but not in vehicle-treated mice, indicating that multiple CEDs of KU60019 at weekly intervals may cause neurological symptoms (Frosina et al, unpublished). Similar, albeit milder, neurological symptoms were observed after multiple i.p. administrations. Mice were injected daily i.p. with 150 nmoles of KU60019 for two weeks/five days a week (total of 10 i.p. injections). Hyperkinesis was observed in treated but not in vehicle-injected animals at the end of treatment (Frosina et al, unpublished). Since pharmacokinetics studies performed by HPLC/MS never showed brain penetration by KU60019 after its i.p. administration (Vecchio et al., 2015; Frosina et al., 2018b), the observed neurological toxicity might possibly be exerted to the meningeal and/or peripheral nervous compartments. Further preclinical studies on the possible neurological effects of multiple ATM administrations could be warranted.

The ATM inhibitor KU59403 has been shown to act as a sensitizer of chemotherapeutic agents inducing DNA strand breaks (Batey et al., 2013). Albeit KU59403 concentrations were detected in normal tissues of treated animals for at least 4 h after delivery, KU59403 was nontoxic alone: KU59403 given as a single agent caused < 2% maximum body weight loss, indicating that enhancement of the efficacy of chemotherapeutic drugs causing DNA breaks can be obtained in xenograft models by combination with KU59403 with little enhancement of toxicity as measured by body weight loss (Batey et al., 2013).

Recent pharmacological studies have shown the potent inhibitory action (cellular IC_{50}: 0.78 nM) and high specificity (more than 10,000 times compared to other similar kinases) of AZD1390 on the ATM protein (Durant et al., 2018). As aforementioned (section “ replace with 3. GB radiosensitization via ATM inhibition”), AZD1390 can be taken orally and has been specifically optimized for the penetration of the BBB as confirmed by positron emission tomography (PET) performed on the brain of monkeys treated with radioactively-labeled AZD1390. AZD1390 blocks the activity of ATM-dependent DDR and amplifies the effect of IR in inducing cell accumulation in G2, micronuclei and apoptosis. In vitro, AZD1390 radiosensitizes glioma and lung tumor cell lines, especially if mutants in p53. In orthotopic models of HGG and pulmonary brain metastases, AZD1390 administered in combination with daily fractions of IR induced tumor regressions and significantly increased animal survival compared to IR treatment alone. However, these data have been obtained on a limited number of rapidly and relatively uniformly growing orthotopic tumors, which only partially mimic the growth characteristics of clinical tumors. Further studies evaluating the efficacy of AZD1390 on multiple orthotopic models of both adult and infant GIC-induced gliomas, which more accurately reproduce in the animal the multiform ways of growth of clinical equivalents, may be warranted. Similarly to former generation ATM inhibitors, the toxicity of AZD1390 is limited. Neither overt behavioral symptoms nor body weight changes were observed in orthotopic tumor animal models treated with 20 mg/kg AZD1390 plus IR (Fig. 4). The concentration of AZD1390 had to be reduced from 20 to 15 mg/kg when a TMZ dose that is the equivalent of that used in the clinic was added (Durant et al., 2018).

4.3. Clinical studies

In order to study the drug pharmacokinetics in humans, a microdose of radioactively-labeled [^{11}C]AZD1390 has been administered to healthy volunteers aged 20–65 years https://clinicaltrials.gov/ct2/show/NCT03215381?term=AZD1390&rank=1. This link could not open directly after clicking All volunteers were preliminarily examined by a baseline brain MRI for eligibility and anatomical delineation of brain regions to be applied in positron emission tomography (PET) analysis. The single intravenous microdose of [^{11}C]AZD1390 was administered by bolus injection in the arm. Blood samples were collected early after the administration and during the subsequent PET imaging. This study has been completed but no results have yet been published.

As a next step, a study to assess the safety and tolerability of AZD1390 given with RT in patients with brain cancer is now recruiting https://clinicaltrials.gov/ct2/show/NCT03423628?term=AZD1390&rank=2. link could not open directly after clicking This multicenter Phase 1 study is being conducted in the United States and in the United Kingdom, and aims to assess safety and tolerability of AZD1390 in combination with RT in brain malignancies. The combination cohorts have been designed to assess escalating cumulative doses of AZD1390 in the presence of 3 different RT regimens.

AZD0156 is a further selective and orally bioavailable ATM inhibitor (Degorce et al., 2016, 2018). AZD0156 exhibits activity in combination with agents inducing DSBs and may synergize with inhibitors of alternate pathways of DNA break repair such as the PARP inhibitor olaparib.

A currently recruiting clinical trial consists of different study modules, each evaluating the safety and tolerability of AZD0156 with a specific combination agent https://clinicaltrials.gov/ct2/show/NCT02588105?term=ATM&draw=3&rank=69. Initially, monotherapy dose escalation is performed to gain an understanding of pharmacokinetics, safety and tolerability of AZD0156 alone. Then dose escalations in combination are explored. An oral formulation of AZD0156 is used. Module 1 explores AZD0156 in combination with olaparib. Module 2 explores AZD0156 in combination with the DSB inducing agent irinotecan. Preliminary results show that hematologic toxicity may be the treatment-limiting toxicity for AZD0156 in combination with those agents (Abida et al., 2018).

5. Toxicity of pharmacologic ATR inhibition

The Ataxia Telangiectasia and Rad3 related protein (ATR) is a further important mediator of the DDR that may be involved in tumor resistance to radio- and chemotherapy. NVP-BEZ235 is a dual PI3K/mTOR inhibitor that also effectively targets ATR with $IC_{50} = 21 \times 10^{-9}$ M in cells. AZD6738 does not target significantly PI3K/mTOR-related kinases but specifically inhibits ATR with $IC_{50} = 74 \times 10^{-9}$ M in cells. Both drugs have been proposed as radiosensitizers of different tumors including GB. Using high performance liquid chromatography/mass spectrometry (HPLC/MS) we have investigated whether the two drugs could cross the BBB and penetrate the brain (Frosina et al., 2018a). We found elevated bioavailability and efficient crossing of the BBB for both drugs and especially for AZD6738. However, a trend towards reduced median survival was observed in mice bearing GIC-driven orthotopic tumors treated with 25 mg/kg body weight NVP-BEZ235 plus RT as compared to mice treated with vehicle plus RT (86 versus 99 days, respectively) while no effect on survival was observed in mice treated with AZD6738. Consistently, Netland and coworkers have reported severe side effects of doses of NVP-BEZ235 higher than 25 mg/kg body weight in both nude rats and NOD/SCID mice bearing orthotopic GB (Netland et al., 2016). The early termination of recent NVP-BEZ235 clinical trials due to elevated toxicity may confirm that safety dosing of NVP-BEZ235 should be thoroughly investigated prior to use in the clinical setting (Carlo et al., 2016; Pongas and Fojo, 2016).

A clinical trial entitled “AZD6738 First Time in Patient Multiple Ascending Dose Study” has been completed https://clinicaltrials.gov/ct2/show/NCT01955668. AZD6738 was administered orally to patients with relapsed/refractory chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL) or B cell lymphomas, in order to determine the safety and tolerability of AZD6738. A starting dose of 20 mg twice daily was escalated in patients up to the maximum tolerated dose. No

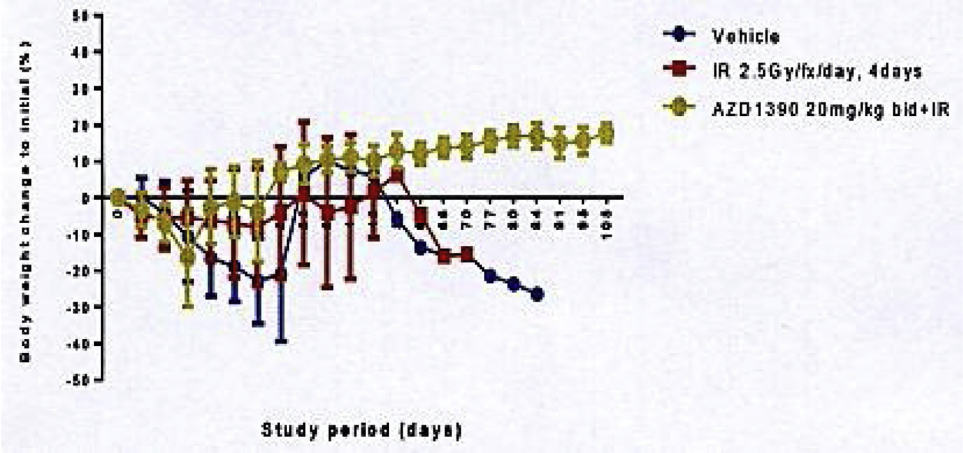

Fig. 4. In vivo toxicology of AZD1390. Body weights of NCIH2228 brain metastasis model mice treated with IR or AZD1390 20 mg/kg + IR (after (Durant et al., 2018) with permission).

results from this trial are yet available.

6. Conclusion

ATM inhibition may be a viable strategy for tumor radio- and chemosensitization. In particular, preclinical efficacy has been demonstrated for some ATM inhibitors as radio- and chemo sensitizers of GIC-driven orthotopic GB. The promise of these molecules is augmented by their limited toxicity after transient administration, as observed both in vitro and in vivo experiments. However, the consequences of multiple and prolonged administrations of ATM inhibitors have not yet been thoroughly investigated in the preclinical setting. This aspect could warrant further studies as a precautionary measure, prior to further ATM inhibitor employment in the clinical environment.

Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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