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Window of Opportunity Clinical Trials to Evaluate Novel Therapies for Brain Tumors

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INTRODUCTION

Glioblastoma (GBM) is the most common primary brain tumor in adults.[1] Despite aggressive surgical resection followed by radiotherapy and temozolomide (TMZ)-based chemotherapy, the median overall survival (OS) remains only 15 months.[2] Unfortunately, over the last few decades, OS has improved by only a few months indicating that there is an urgent need for more effective therapies.

Following pre-clinical development, new anticancer therapeutics undergo a series of progressively complex and larger prospective clinical trials whose goals are 1) to identify the maximal tolerated dose (MTD) of a new agent that can be given without serious side effects (Phase I), 2) to determine whether the agent works against particular tumors at this dose (Phase II), and to compare the safety and efficacy of the new agent with the current standard of care (Phase III).[3, 4] Unfortunately, for most brain tumors, particularly GBM, many new therapies fail in large and expensive phase III clinical trials, despite perceived success in earlier phase I and phase II trials.[5–8] Of equal concern, many agents that seem to be efficacious in preclinical animal studies are dismissed as ineffective after phase II testing without knowledge of why the agent did not work against human GBMs.[9] As a result, there have been few new therapeutics for GBM approved by the Food and Drug Administration (FDA) in the past 30 years.[5]

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Multiple factors likely contribute to the overall low number of agents that successfully pass through the translational continuum and become new therapeutics for GBM, including the highly resistant phenotype of GBM, the uniqueness of the CNS microenvironment, the limitations to delivery imposed by the blood brain barrier (BBB), and the lack of preclinical models that recapitulate the human disease and predict clinical success.[10–14] However, this poor outcome is also due at least in part to the current methods used to evaluate new brain tumor therapeutics in patients (Figure 1). Specifically, traditional Phase I dose escalation trials typically identify the MTD based purely on assessments of systemic toxicity, such as nausea, vomiting or diarrhea.[15] Likewise, phase II trials usually determine efficacy based on indirect measures, typically radiographic response, progression free survival (PFS) and OS (Figure 1).[16] Indeed, traditional early stage trials of brain tumor therapeutics do not include direct assessments of tumor tissue, and therefore, do not determine whether the agent is capable of doing what it is intended to do in GBM tumors in human patients, which is arguably the most important endpoints of early stage drug development. As a result, agents that do not show radiographic response when used as monotherapy are dismissed, although they may in fact hit their target in the tumor cells and could be used in combination with other agents.[17] Alternatively, agents, such as bevacizumab, that produce radiographic responses as measured by changes in contrast enhancement on MRI may be deemed effective and move on to large phase III trials, although they have little ability to inhibit tumor growth.[6, 18]

To address these shortfalls and expedite the discovery of new effective agents for GBM, over 20 years ago, concurrent with the development of molecularly targeted therapeutic agents, and building on proposals in other solid tumors[19], we[20] and others[21, 22] proposed modifications to traditional early phase clinical trials for the treatment of GBM. The primary goal of these modifications was to demonstrate that the new agent was able to do what it was intended to do, namely, that it crossed the BBB and entered the brain tumor, hit its molecular target, and caused cell death and/or induced physiologically important changes in the tumor microenvironment (e.g., enhance anti-glioma T cell populations[23]). To achieve this goal, it was proposed that the new therapeutic agent should be administered pre-operatively to patients with surgically resectable tumors, the tumor be removed at surgery, and that the post-treatment tumor specimen be analyzed for drug penetrance and biological activity, essentially recapitulating what is done during preclinical testing in the laboratory (Figure 1). These trials were originally designated as “biological endpoint” trials because changes in the molecular biology of the tumor defined outcome.[20, 21, 24] However, soon these trials became known a “window of opportunity” trials because surgical resection provided an opportunity to obtain a post-treatment specimens that could give a window into the molecular effects of the agent on the tumor.[25] Over the past 20 years, window of opportunity trials have become increasingly accepted as an important component of the development of novel therapeutics of solid tumors, including brain tumors like GBM, and are considered by many as the gold standard of early stage clinical drug development.[22]

Execution of an effective window of opportunity trial for brain tumors is particularly complex due to the difficulties inherent in obtaining tumor tissue from the brain. Therefore, in this review, we define what constitutes window of opportunity trials. We discuss novel designs for incorporating biological endpoints into early stage trials in the context of

glioblastoma, and through examples of successful window of opportunity trials, we illustrate the power of window of opportunity trials in the development of novel therapeutics for GBM.

Defining features of a window of opportunity trial

Window of opportunity trials are designed specifically to determine the extent to which a new therapeutic agent hits the molecular target against which it is designed and/or appropriately modulates the tumor microenvironment for therapeutic effect. When combined with traditional metrics of safety (e.g., clinical toxicity) and efficacy (e.g., radiographic response), data from window of opportunity trials provide critical molecular or cellular information about why agents succeed or fail during clinical trials. These results guide modifications early in the clinical trial continuum, including making go/no go decisions prior to expensive and time-consuming late stage clinical trials. Window of opportunity trials incorporate several critical features that must be considered in the trial design, including tissue acquisition, molecular assays, and dose schedules (Figure 1).

Tissue Acquisition—The most important element of a window of opportunity trial is tissue acquisition after drug treatment. For brain tumors, tissue acquisition is a significant hurdle because of the potential complications associated with invasive brain procedures.[26] Tissue can be obtained either through stereotactic biopsy or through open craniotomy and tumor resection. Stereotactic biopsy has the advantage that it is minimally invasive and affords the opportunity for serial biopsies before and after drug treatment, which is highly desirable as the pretreatment sample provides a baseline untreated (control) tissue (see below); however, the tissue samples are often small, limiting the number of assays that can be performed, and under-evaluating the true heterogeneity of the tumor. Alternatively, open craniotomy and surgical resection often provides large amounts of tumor for analysis. Given the significant spatial heterogeneity of different clonal sub-populations within GBM,[27] optimal specimens for analyses are achieved using resection techniques in which the tumor is removed by circumferential dissection as previously described (Figure 2).[28] This approach not only provides a large tumor sample for multiple analyses, but also preserves the tumor architecture, permitting assessments across the various tumor zones, including areas of solid tumor, infiltrating tumor cells and assessing the impact of the new therapy on different clones of tumor cells residing in different niches within the tumor such as hypoxic areas, which may contribute to treatment resistance[29]

Because of the complexities of including craniotomies in trial designs, many window of opportunity trials have optional arms for tissue acquisition. However, the most effective window of opportunity trials incorporates tumor acquisition specifically into clinical trial protocols. For phase I window of opportunity dose escalation trials, inclusion of two arms, a standard treatment arm and a biological endpoint arm, is optimal in our experience (Figure 3).[24, 30] In the first arm (standard treatment arm), drug is given, and patients are simply followed for standard toxicity measures, providing traditional assessments of drug induced toxicity. In the second arm (biological endpoint or window of opportunity arm), patients are treated with drug and then undergo craniotomy and tumor resection, thereby providing a post-treatment biological specimen for analysis of drug induced changes. We successfully

used this strategy in a trial of an adenovirus mediated p53 gene therapy (Ad-p53) and in another trial of Delta-24-RGD oncolytic virus.[24, 30] In these trials, both arms were enrolled at each dose cohort, with arm 2 (biological endpoint arm) enrolling after arm 1 (standard treatment arm) (Figure 4; Strategy A). An alternative strategy, which is more cost efficient, is to enroll successive cohorts in arm 1 (standard arm) until the MTD is reached. Then, once the MTD is determined, patients are enrolled into arm 2 (biological endpoint) (Figure 4; Strategy B). Whereas the former strategy defines the biological outcome at each dose cohort, the latter strategy first defines the MTD and then assesses whether the MTD dose results in target engagement/alteration. In our most recent window of opportunity trial[31], we have followed this latter strategy as it allows for faster dose escalation, is less costly, and requires fewer patients.

Molecular Assays—The second critical component of a window of opportunity trial is a predefined molecular assay that assesses the desired molecular outcome. Determining the optimal assay is paramount to the success of any window of opportunity trial. One of the most basic assays that should be included in nearly all window of opportunity trials of drugs is a simple assay of drug levels in the tissue. This is particularly important in brain tumors where the BBB can prevent drug from reaching the tumor. Indeed, a major goal of window of opportunity trials is to simply demonstrate therapeutic levels of the drug in the tumor after the drug is delivered at the dose and schedule that will be moved forward into later stage trials. Because the BBB is difficult to assess in animal models[32], the simple documentation that adequate concentrations of drug are present in a tumor is an important outcome of window of opportunity trials.

Equally important, is incorporating molecular assays that prove that the drug is interacting with its target. Ideally, the target of the agent is known and an assay for assessing engagement with that target is available. For example, determinations of the effectiveness of tyrosine kinase inhibitors may require assays that document inhibition of phosphorylation of the target receptor.[17] Likewise, the effectiveness of an immunotherapeutic agent may be shown by assays that assess increases in anti-tumoral T-cells.[23] Importantly, these assays must be applicable to clinical tissue specimens. Assays that assess target inhibition on tissue slices (e.g., immunohistochemistry (IHC)) are preferred as they provide molecular information in the context of spatial distribution.[24, 30, 33] Nevertheless, most molecular assays that are developed in preclinical laboratory testing can be applied in window of opportunity trials, incldiung western blotting[17], IHC[24,30], in-situ hybridization,[27] mass cytometry (CYTOF), and single cell RNA sequencing[34].

In this context, it is important that a major goal of preclinical animal studies of new targeted agents is the development of assays of target engagement that can be translated to clinical specimens. Indeed, most preclinical studies of new agents use animal models to prove efficacy by determining whether the agent improves animal survival.[35] However, an equally important application of preclinical animal models is developing critical assays that can be translated to clinical specimens. For example, in our work on Delta-24-RGD, an oncolytic virus, we developed a tissue-based assay using intracranial tumors from mice in which we documented the presence of active virus based on immunohistochemistry using antibodies against viral proteins (hexon and E1A.[30, 36] We showed in our animal models that

intratumoral injections of Delta-24-RGD resulted in three concentric zones indicative of virus propagating through the tumor: a central zone of necrosis (where the virus had killed cells), a surrounding zone of cells that stained positively for hexon or E1B protein on IHC (where the virus was actively replicating in tumor cells) and a third zone of tumor cells (where the virus had not reached yet) (Figure 5).[36] This immunohistochemical assay and the zones of activity were translated to our clinical specimens and used to prove that Delta-24-RGD could infect and replicate in human tumors from patients with glioblastoma.[30]

Assays that measure direct effects of a drug are preferable to assays that measure indirect effects or downstream effects. For example, if a drug is known to inhibit a particular molecular target, such as by blocking phosphorylation, the ideal assay would measure the phosphorylation status of the target (e.g., by western blotting).[17] Measurements of downstream effects, such as induction of apoptosis (as measured by cleaved caspase-3 activity) are less desirable.[37]

In this context, the heterogeneity of molecular events within any tumor mandates careful consideration of control tissue for comparison of molecular outcomes in any window of opportunity clinical trial. Although some molecular outcomes may not require a control (e.g., level of a drug in a tissue or identification of a therapeutic oncolytic virus in a tumor), baseline levels of most molecular targets vary greatly across tumors. For example, assaying inhibition of phosphorylation of a tyrosine kinase, such as EGFR, requires knowing the baseline level of phosphorylation before treatment.[17] Consequently, although inclusion of controls is not standard in traditional phase I or phase II clinical trials, defining the baseline levels of a molecular target is critical for the success of a window of opportunity clinical trial. Although comparing an untreated specimen to a treated specimen is ideal, acquiring an untreated specimen may be difficult in patients with brain tumors as a biopsy before and after treatment would be needed. Therefore, several novel avenues for acquiring control tissue have been proposed (see below) and many window of opportunity have exploited these methods.

Dose Schedules—The last critical element of a window of opportunity trial is the dose and schedule of the experimental agent that is given to the patient. The defining feature of window of opportunity trials is that the delivered dose is a therapeutic dose that has the potential to be effective against the tumor. Using a therapeutic dose distinguishes window of opportunity trials from so called Phase 0 trials. Unlike window of opportunity trials, Phase 0 clinical trials are carried out very early in drug development.[38] Here, investigators give a sub-therapeutic dose of an investigational agent without diagnostic or therapeutic intent.[39] The primary goal of these studies is to assess the pharmacokinetics (PK) and pharmacodynamics (PD) of new agents that have never been tested in humans.[40, 41] If these agents are found to meet the PK and PD thresholds, these candidate drugs then proceed to phase I dose escalation studies to assess safety and toxicity.[39] Whereas giving subtherapeutic doses of a drug and assaying blood levels of the agent may give valuable information about drug kinetics, Phase 0 trails offer little value in terms of defining whether therapeutic levels of a drug enter a tissue or hit its target, particularly for brain tumors where subtherapeutic levels are not likely to cross the BBB. In window of opportunity trials, however, therapeutic doses of new agents are administered with a biological endpoint as the goal.

In addition to the dose, a critical element of window of opportunity trials is deciding the length of treatment prior to tissue acquisition and the amount of time after treatment that the tissue will be acquired. Given that the goal of these trials is to determine the extent to which the drug hits its target or causes a desired molecular or cellular effect, consideration should be given to treating the patient for an extended period of time in order to maximize the potential molecular effects of the experimental agent. For example, lapatinib, an EGFR inhibitor, is typically dosed on a twice daily schedule, and so in a recent window of opportunity trial this drug was given twice daily for seven days before the tumor was resected.[17] This strategy assured that the drug levels detected in the tumor were clinically relevant. In this context, a common approach is to treat for a long enough period of time so as to reach the steady state drug level (typical five half-lives of the agent) prior to tissue acquisition. Similarly, window of opportunity designs must carefully consider how much time should elapse between the last dose and tissue acquisition. Indeed, the molecular effects of the experimental agent may be delayed relative to drug delivery, particularly when the assayed effect is downstream of the molecular target. For example, in a currently ongoing trial assessing the ability of bone marrow stem cells to deliver Delta-24-RGD to brain tumors after intra-arterial injection, post treatment tissue is acquired 14 days after the injection based on preclinical studies showing it takes this much time for the cells to arrive in the tumor, release the virus, and then maximally infect the tumor cells.[31]

Incorporating window of opportunity trials into early phase trials of brain tumors.

Given the multiple considerations discussed above, several strategies have been suggested for tailoring phase I and phase II trials to incorporate biological endpoints and thereby meet the goals of window of opportunity trials for brain tumors. We have previously proposed several approaches which are worthy of review, and it is anticipated that new approaches will emerge to meet the needs of particular experimental agents.[20]

Phase I trials.—The goal of Phase I clinical trials is to determine the MTD or the recommended phase II dose (RP2D) that can be given to a patient without causing undue toxicity. Incorporation of biological endpoints in a window of opportunity phase I trial provides the further determination of whether the new agent influences the molecular endpoint being targeted, and therefore proves that the MTD or RP2D is also an effective biological dose (EBT). Indeed, in some cases the EBD may prove to be lower than the MTD, although such examples are rare. We have previously suggested several strategies of carrying out effective Phase I window of opportunity clinical trials. [20]

In the ideal design (Figure 6, Design A), patients with surgical accessible lesions undergo a stereotactic biopsy to collect pre-treatment tissue for baseline molecular analysis using the same assays that will be assessed after treatment After this baseline (control) tissue is obtained, the experimental therapeutic is administered based on the preplanned dose and schedule. At the completion of the drug treatment, a craniotomy and *en bloc* tumor resection is performed (alternatively another biopsy may be performed if tumor resection is not feasible). The post treatment tumor specimen undergoes molecular analyses to determine tissue pharmacokinetic and drug-related molecular changes. After the patient recovers from the surgery, the agent is given (typically at the same dose given before surgery) in order to

determine clinical toxicity according to standard phase I criteria. This 2-stage design has the advantage that it allows comparisons to be made between untreated (control) and treated specimens from the same patient. However, the need for two procedures makes this design less desirable to many patients. A version of this strategy was used in our recent clinical trial of DNX-2401 oncolytic virus therapy for recurrent GBM, [24, 30] in which post treatment specimens were compared with untreated (control) tissue taken at the initial resection from the same patient (albeit not immediately before treatment).

To minimize the number of biopsies, several alternatives are possible. One strategy (Figure 6, Design B) is to randomize patients with surgically accessible lesions to a group that receives the therapy versus a group that does not receive the therapy or that receives a placebo. Patients in both groups undergo craniotomy and en bloc tumor resection. Molecular analyses are performed on all tumor specimens with the specimens from the untreated/placebo group serving as controls for the treated group. Following surgery, both groups receive the agent and are followed for clinical toxicity using standard phase I criteria. In this strategy the number of patients entered depends on the test-variability of the molecular analyses, which will define the number of patients needed to achieve statistical significance. This strategy has the advantage of requiring only one surgical intervention for each patient, however, it suffers from requiring the use of statistical methods to determine the variability in the baseline levels of the targeted molecule and to determine whether there is an overall impact of the therapy on the molecular target. Another alternative is to use banked specimens as the untreated control group for comparison to the treated group, exemplified in the Phase II clinical trial assessing pembrolizumab (PD-1 inhibitor) in recurrent glioblastoma patients[42](Figure 6, Design C). This strategy eliminates the need for randomizing patients to drug versus no drug treatment and therefore requires less patients, but is limited by its reliance on non-contemporaneous historical controls that may have been handled and processes differently from the prospectively obtained post treatment specimens.

Phase II trials—Design strategies similar to those proposed for phase I trials can also be applied to phase II trials, again recognizing that these phase II trials must retain the goal of determining clinical efficacy while incorporating biological endpoints. Similar to the ideal phase I strategy, in the ideal phase II approach, patients undergo a stereotactic biopsy before treatment, after which they are treated with the therapeutic agent using the dose defined in phase I. After treatment with the therapeutic agent, patients undergo surgical resection providing a post-treatment specimen that can be compared with the pretreatment specimen to determine whether the agent hit its target or altered the tumor microenvironment to the degree established in the phase I setting. In this approach, the molecular endpoint can be used as a response criterion to define the efficacy of an agent, such that agents that repeatedly hit their target or induce the desired molecular effect are deemed efficacious [30]. This efficacy analysis can be extended further by administering the experimental agent after surgical resection and by then following the patient to determine PFS or OS, based on MRI analyses of tumor recurrence. This is exemplified in the window of opportunity phase II trial assessing the efficacy of Lapatinib (EGFR inhibitor) for recurrent glioblastoma.[17] Here, patients with surgically resectable recurrent GBM were treated with lapatinib (750 mg orally twice daily) for seven days before surgical removal of the tumor. The surgical specime was

analyzed for levels of lapatinib and for inhibition of EGFR phosphorylation. After recovery from surgery patients were treated with daily lapatinib until tumor recurrence to assess the impact of the drug on PFS. Since the tumor is largely removed at surgery, assessments of radiographic response may be difficult in this design. Therefore, like phase I trial, these phase II trials with biological endpoints may include a “standard treatment” arm in which no surgical intervention is undertaken so that the traditional endpoints of radiographic response, PFS, and OS can be assessed independent of any surgical intervention[30]. In this arm, a pretreatment biopsy may be undertaken to determine the extent to which the molecular target is present in the tumor at baseline. This information can then be used to define whether the presence of the target correlates with higher rates of radiographic response, longer PFS and longer OS compared with tumors that do not have the target.

In addition to this ideal phase II design, a more cost-effective approach for a window of opportunity phase II study is to randomize patients with surgically accessible lesions to either receive the experimental agent or to receive placebo/no drug, and then to perform a craniotomy and en bloc resection, thereby providing specimens for molecular analyses. In this scenario the placebo/untreated specimens serve as the control for the treated specimens. Biological efficacy is based on observing a statistically significant higher number of patients in which the target is hit in the treatment group compared with the control group. After the craniotomy for resection, all the patients are treated with the experimental agent and followed for tumor recurrence to define PFS and OS.

When done well, these window of opportunity phase II designs add meaningful biological data to the standard clinical outcome data, and enhance decision-making around whether the agent should be further studied. In this context, agents that clearly hit their molecular target in the majority of patients in the window arm of the trial and show both clinical response and extension of PFS/OS in the standard arm are likely to be efficacious and should be moved to phase III trials. In contrast, agents that do not hit their target and do not show responses should likely be abandoned, although the window of opportunity arm of the trail may provide insight at the molecular or cellular level into the cause of the failure. For example, the biological studies may reveal that the drug levels were below those needed to kill tumor cells, or the drug may have inhibited the target only partially. These insights may lead to continued pursuit of the target through the development of more effective agents rather than completely abandoning the target. Potentially more interesting are agents that may hit their target in biological studies, but do not induce clinical responses. Although such a result may suggest that the target is not an independent driver of tumor growth, it may also suggest that the experimental agent may be effective when combined with other agents that hit alternative tumor supportive pathways, thus spurring the rational development of the agent in combination trials.

CONCLUSIONS

The clinical development of new therapeutic agents for GBM could be advanced through improvements in the design of standard phase I and II clinical trials. Window of opportunity trials are such an improvement and should routinely be incorporated into standard early phase clinical trials for GBM (and all brain tumors) because they provide a unique

opportunity to capture the complex cellular and molecular changes induced by molecularly targeted agents, thereby providing information about the biological activity of the agent at its MTD, arguably the most important outcome of any early stage trial. Window of opportunity trials are based on several fundamental principles: acquisition of post-treatment tumor specimens, a clinically applicable assay to detect alterations in molecular targets post-treatment, pre-treatment or untreated control specimens that provide a baseline level of activity against which post treatment changes can be compared, and therapeutic dosing schedules. Incorporation of molecular endpoints (pre-and post-treatment), and advanced histologic techniques that maintain the integrity of molecular endpoints during window of opportunity phase I/II clinical trials provide avenues for assessing the distribution of these agents within tumors and directly determining the extent to which these agents actually modify the target for which they were designed to interact, especially in the BBB-protected CNS. As more studies incorporate molecular endpoint concepts in window of opportunity trials, we anticipate uncovering more rationale combinations of therapies that work synergistically against a heterogenous and resilient disease like glioblastoma.

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KEY POINTS:

1. Window of opportunity clinical trials, also called biological endpoint trials, are designed to provide molecular or cellular evidence from post-treatment human specimens that an experimental therapeutic is capable of entering tumor cells and inducing the desired molecular or cellular changes, which it was designed to produce.
2. When combined with classic phase I or phase II trial endpoints, which define the maximal tolerated doses and a clinically effective dose, window of opportunity trials prove that these doses are also biologically effective doses.
3. Window of opportunity trials are based on three requirements: acquisition of post-treatment tumor specimens, predetermined assays to detect alterations in defined molecular targets in these post-treatment tumor samples, and control untreated tissues against which the molecular changes in the post-treatment specimens can be compared.
4. When standard phase I and II clinical trials are designed to include window of opportunity components, investigators gain valuable insights into why molecularly targeted agents do or do not show clinical benefit in GBM.

SYNOPSIS

Despite significant improvement over the last decade in our understanding of the molecular underpinnings driving glioblastoma (GBM), there has been minimal improvement in the overall survival of patients. This poor outcome is due, at least in part, to the traditional designs of early phase (phase I and phase II) clinical trials, which focus on clinical assessments of drug toxicity and response, and typically do not assess biologically relevant endpoints that determine whether the new drug modifies the target against which it was designed to interact. Window of opportunity trials (aka, biological endpoint trials) overcome this shortcoming by assessing durg-induced on-target molecular alterations in post-treatment human tumor specimens. While there have been many window of opportunity trials for other cancers, few have been performed for GBM due to the complexities around acquiring tumor specimens in brain tumor patients. In this review, we provide an overview of window of opportunity trials, including novel designs for incorporating biological endpoints into early stage trials in the context of brain tumors, and examples of successfully executed window of opportunity trials for GBM.

CLINICS CARE POINTS

Window of opportunity clinical trials provide tissue for assessment of the biological effects of therapies on their intended target.

Window of opportunity clinical trials for brain tumors evaluate whether systemically admin-istered drugs cross the blood brain barrier at biologically effective levels.

Window of opportunity clinical trials for brain tumors could delineate responders from non-responders based on biological bio-markers within tumors.

Future clinical trials for brain tumors should incorporate a Windows of Opportunity arm to assess biological efficacy of new agents.

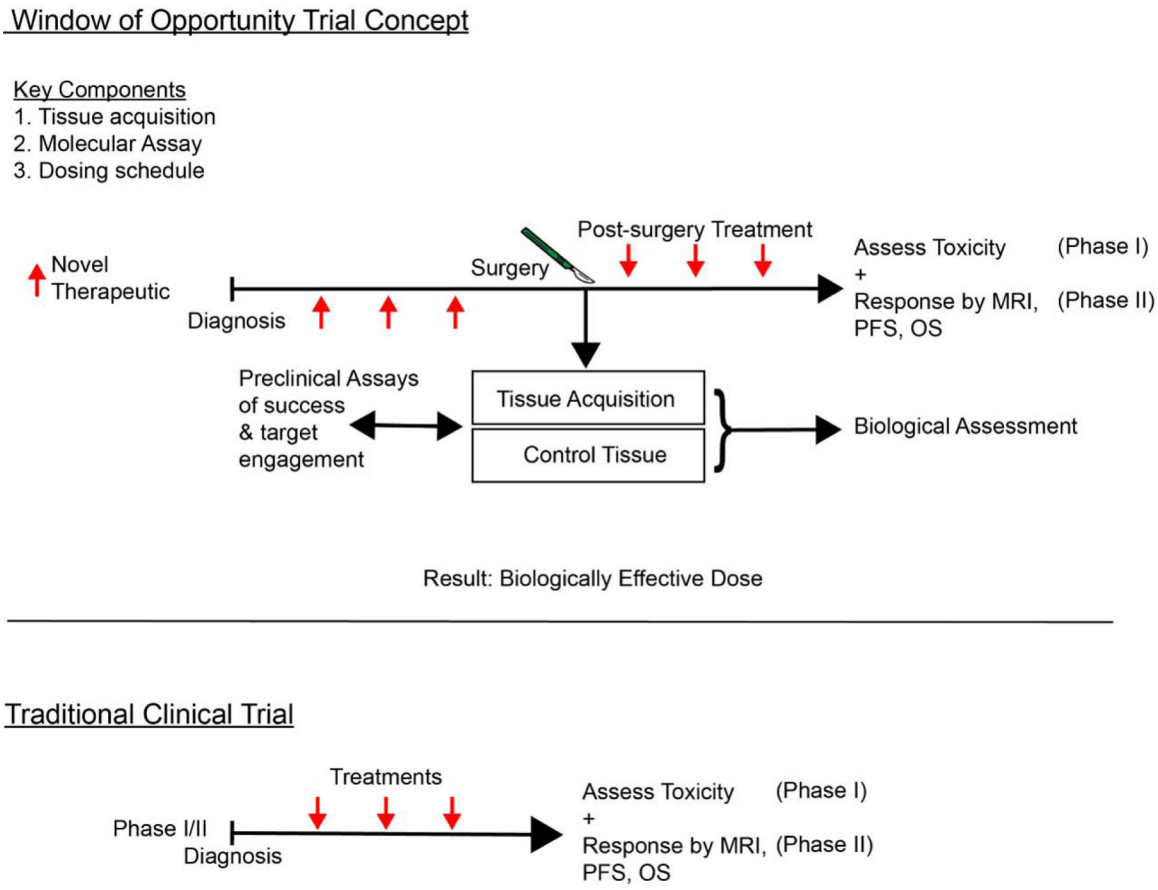

Figure 1. Schema comparing Window of Opportunity Trial concept to Traditional Clinical trials.

In window of opportunity trials, following diagnosis, novel therapies are administered prior to tissue acquisition during surgery. Biological assessment is performed on both treated tissue and an untreated or control tissue to determine if the novel therapy modified its intended target within the tumor (Biological endpoint). This assists with determination of the biologically effective dose. Following surgery, the novel therapy can be continued to determine the maximum tolerated dose (MTD). In traditional clinical trials, novel therapies are administered following diagnosis. MTD and clinical response are assessed without knowledge of the target modification within the tumor.

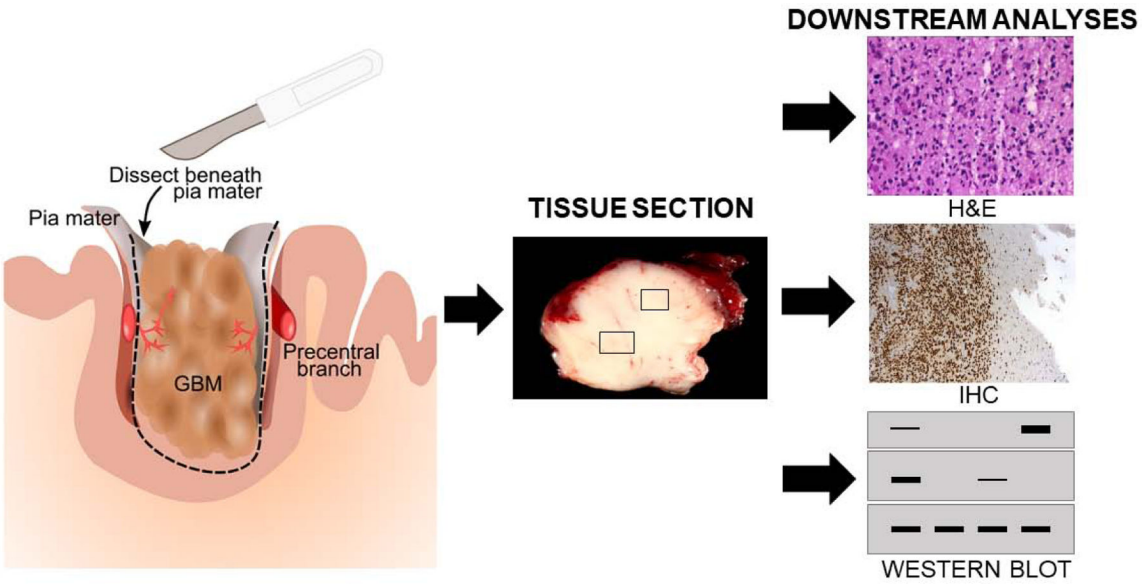

Figure 2. Preserving tumor architecture for analysis.

Enbloc tumor resection using a subpial approach preserves tissue architecture for downstream biological analysis including hematoxylin and eosin (H&E) stains, immunohistochemistry (IHC) and western blot analysis.

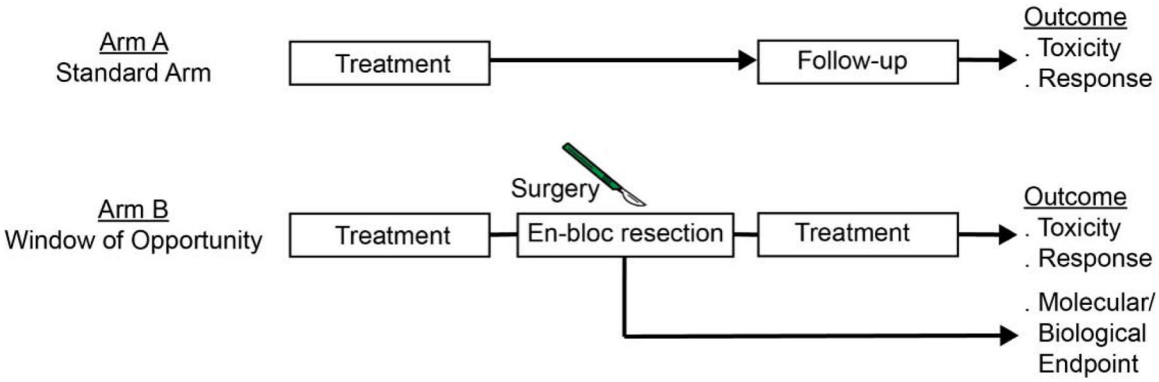

Figure 3. Strategy for incorporating Window of opportunity trail design into standard early phase clinical trials.

The Window of oppporunity component can be achieved by designing a separate arm as part of the standard trial. Specfically, Arm A represents the standard arm in which patients are treated with the novel therapeutic and standard clinical outcomes are measured. Patients with resectable tumors are enrolled into Arm B, which is design to provide post-treatment specimens for analysis.

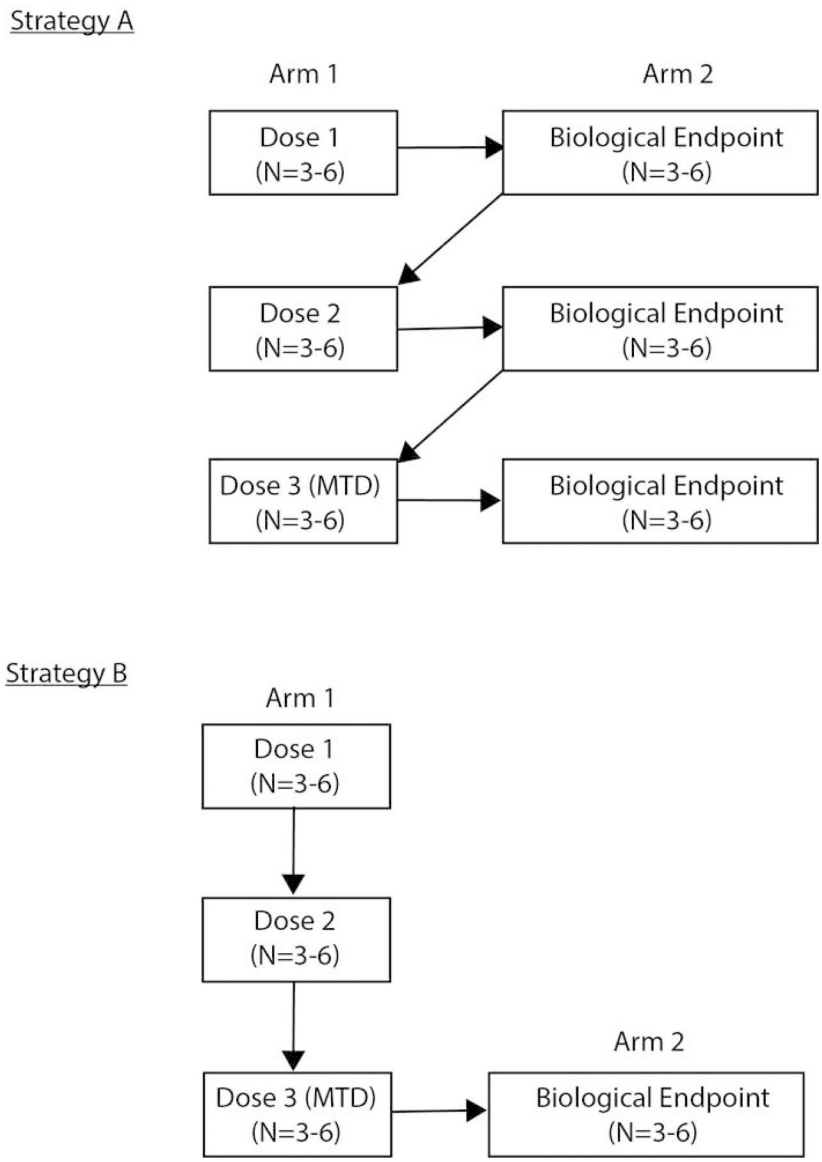

Figure 4. Strategies for dose-escalation during Window of opportunity trials.

In strategy A, both arms are enrolled at each dose cohort, with arm 2 (biological endpoint arm) enrolling after arm 1 (standard treatment arm). An alternative strategy (B), which is more cost efficient, is to enroll successive cohorts in arm 1 (standard arm) until the MTD is reached. Then, once the MTD is determined, patients are enrolled into arm 2 (biological endpoint).

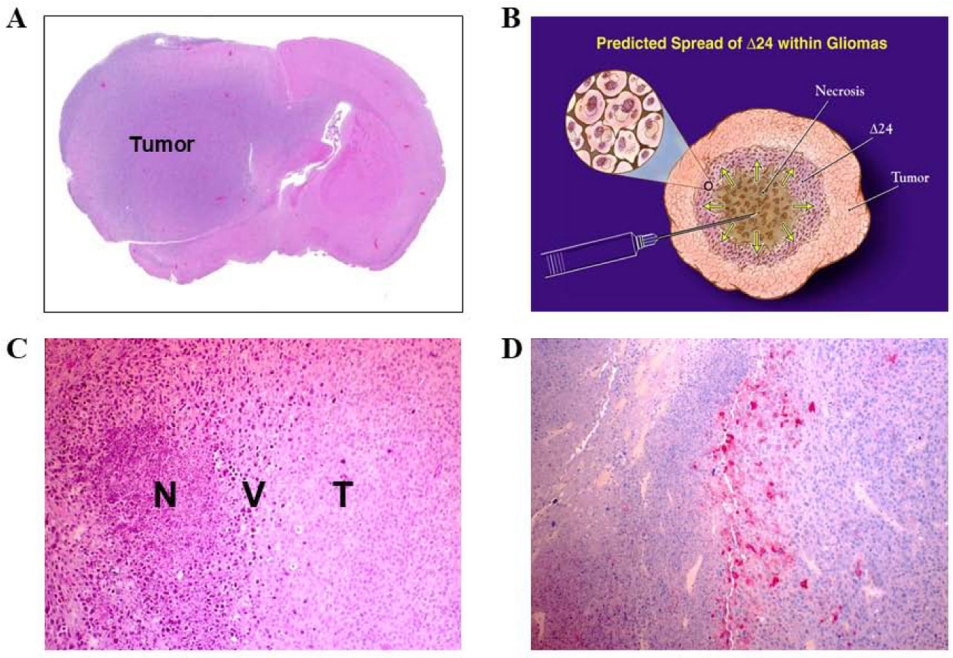

Figure 5. Pre-clinical models for evaluating Delta-24 RGD prior to Window of Opportunity Clinical trials.

Preclinical animal models are important for developing assays that can be translated to clinical trials. For example, in the development of Delta-24-RGD, human gliomas were grown in pre-clinical mouse model as shown by H&E stains of mouse brains (A). To develop an assay that would prove viral replicaton and that could be transted to the clinic, we reasoned that there would be three zones of viral replication (C) when animals were sacrificed several days after intratumoral injection: a central zone of necrosis (N, where the virus had killed cells), a surrounding zone of cells that stained positively for hexon or E1B protein (V, where the virus was actively replicating in tumor cells) and a third zone of yet-to-be-infected tumor cells (T, where the virus had not reached yet). These zones were demonstrated in mouse tumors based on H&E (D) and after staining for E1A (red staining in E). This assay was used in a Phase I trial of this agent

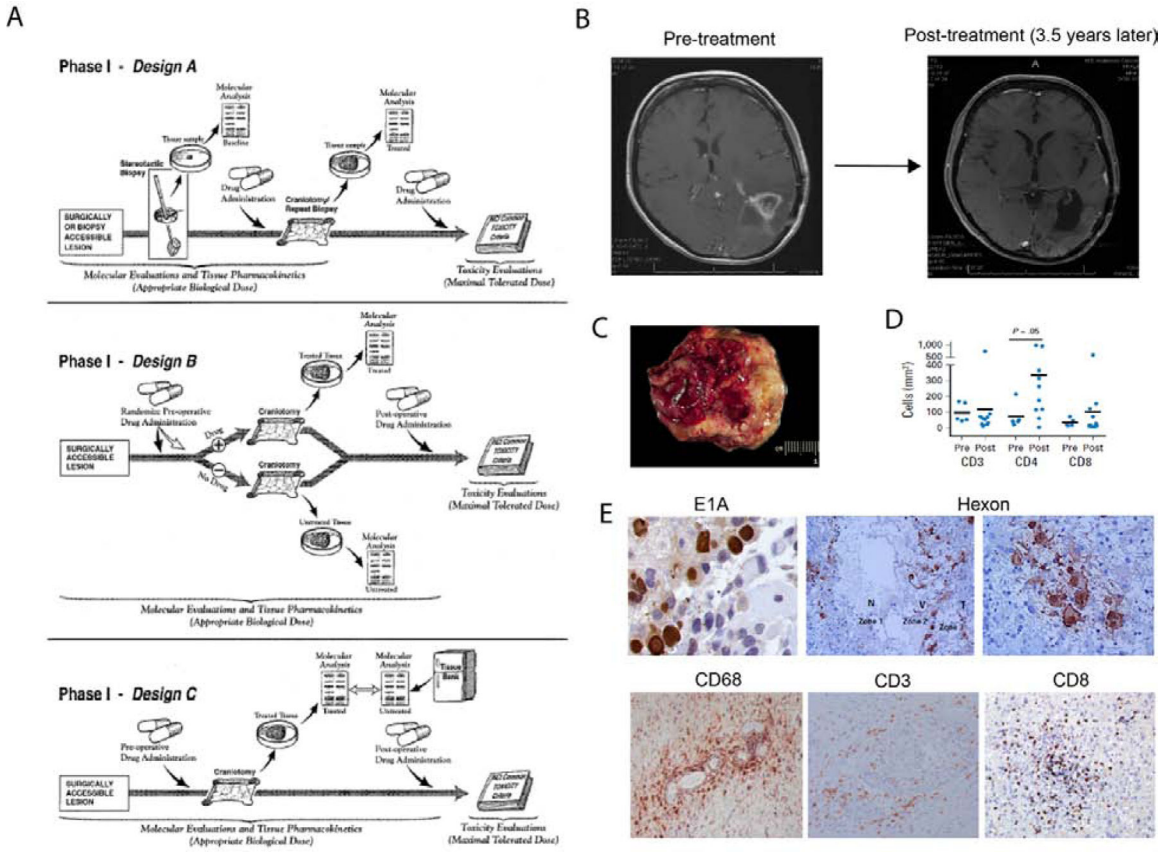

Figure 6. Proposed designs for phase I clinical trials of agents for which molecular targets can be tested and Window of opportunity trial assessing the oncolytic virus, Delta-24-RGD.

Window of opportunity trials can take on several designs (A). Optimal design (Design A). A biopsy can be used to determine the baseline value of the target prior to treatment. Patients are then treated with drug, and the effects of the agent on the tumor are determined in a posttreatment surgical specimen. In design B, which avoids pretreatment biopsy, patients are randomized to receive drug or not. Posttreatment tumor reaction allows comparisons to be made between untreated specimen (controls) and treated specimens. In design C, control specimens are obtained from specimens in a tumor bank. Execution and outcome of a window of opportunity trial for Delta-24-RGD oncolytic virus is shown in B-E. (B) MRI with contrast of long term survivor 3.5 years following Delta-24 treatment and surgery. (C) Photomicrographs of sections from en bloc resection specimens taken 14 days after virus injection. (D) Biological response based on quantitative analyses of CD3+, CD4+, and CD8+ cell infiltration in pretreatment (pre; n = 5) and post-treatment (post; n = 10) tumor specimens. The mean values are noted by horizontal bars; although CD3+ changes were not evident, both CD4+ and CD8+ cells increased after treatment, with increases in CD4+ cells reaching statistical significance. (E) Immunohistochemical staining for viral E1A protein (left), which is a marker of viral infection, and for viral hexon protein (right), which is a marker of replication. E1A immunostaining (left) is primarily intranuclear, as would be expected for actively infecting virus. Immunohistochemical staining for CD68+ Macrophages, CD3+ and CD8+ T-cells. ([A] *From* Lang FF, Gilbert MR, Puduvalli VK, et al. Toward better early-phase brain tumor clinical trials: a reappraisal of current methods and

proposals for future strategies. Neuro Oncol. 2002;4(4):274; with permission. [B–E] *From* Lang FF, Conrad C, Gomez-Manzano C, et al. Phase I study of DNX-2401 (Delta-24-RGD) oncolytic adenovirus: replication and immunotherapeutic effects in recurrent malignant glioma. J Clin Oncol. 2018;36(14):1419–27; with permission.)