# Quantification of endothelial cell–targeted anti–Bcl-2 therapy and its suppression of tumor growth and vascularization

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## Abstract

**Proapoptotic and antiapoptotic proteins in the Bcl family are key regulators of programmed cell death. It is the interaction between these molecules that determines cellular response to apoptotic signals, making them attractive targets for therapeutic intervention. In recent experiments designed to study tumor angiogenesis, Bcl-2 upregulation in endothelial cells was shown to be a critical mediator of vascular development. In this article, we develop a mathematical model that explicitly incorporates the response of endothelial cells to variations in proapoptotic and antiapoptotic proteins in the Bcl family, as well as the administration of specific antiangiogenic therapies targeted against Bcl-2. The model is validated by comparing its predictions to *in vitro* experimental data that reports microvessel density prior to and following the administration of 0.05 to 5.0 μmol/L of BL193, a promising small molecule inhibitor of Bcl-2. Numerical simulations of *in vivo* treatment of tumors predict the existence of a threshold for the amount of therapy required for successful treatment and quantify how this threshold varies with the stage of tumor growth. Furthermore, the model shows how rapidly the least effective dosage of BL193 decreases if an even moderately better inhibitor of Bcl-2 is used and predicts that increasing cell wall permeability of endothelial cells to BL193 does not significantly affect this threshold. A critical challenge of experimental therapeutics for cancer is to decide which drugs are the best candidates for clinical trials. These results underscore the potential of mathematical modeling to guide the development of novel antiangiogenic therapies and to direct drug design. [Mol Cancer Ther 2009;8(10):2926–36]**

## Introduction

The Bcl family of proteins have been identified as crucial mediators of apoptosis, a form of cell death in which a programmed sequence of events leads to the disintegration of cells without releasing harmful substances into the surrounding tissue. There are antiapoptotic Bcl proteins including Bcl-2 and Bcl-$X_L$, as well as their proapoptotic counterparts Bax, Bad, Bak, and Bid. These proteins are constitutively expressed within cells and it is the interaction between these molecules that determines cellular response to apoptotic signals such as intracellular damage and deprivation of or exposure to cytokines and growth factors (1–3). Bcl proteins all differ slightly in size and intracellular location. Bcl-2 is a 26-kDa protein (4), whereas Bcl-$X_L$ is reported to be slightly smaller (5). They are located mainly in the nuclear envelope, parts of the endoplasmic reticulum, and outer mitochondrial membrane (4, 6). Bax is a 21-kDa protein (7), and is localized to the outer mitochondrial membrane (6). The 18.4-kDa protein Bad can be found on the mitochondrial outer membrane, as well as the cytoplasm,[4] whereas the 23.4-kDa protein Bak is a single-pass membrane protein.[4] Bid is a 22-kDa protein and resides in the cytoplasm.[4]

The core component of cellular apoptotic machinery is a family of proteases called caspases (1). Caspase activation can be initiated either extracellularly (extrinsic) or intracellularly (intrinsic). The extrinsic pathway triggers apoptosis in response to the ligation of cell death receptors, which include tumor necrosis factor receptor 1, Fas (CD95/Apo1), DR4, and DR5. Upon activation by their respective ligands, including tumor necrosis factor-α, Fas-ligand, tumor necrosis factor–related apoptosis-inducing ligand/Apo2L, the intracellular domains of the death receptors, also known as death domains, bind to the adaptor protein Fas-associated death domain. This results in the recruitment and activation of caspase 8 and/or caspase 10, leading to the assembly of the death-inducing signaling complex, ultimately inducing cellular apoptosis (8, 9).

The intrinsic pathway triggers apoptosis in response to DNA damage, defective cell cycle, hypoxia, cellular damage induced by most chemotherapy agents, or irradiation and other types of severe cell stress (8). Cell death occurs due

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[4] Data obtained from the GeneCards database at http://www.genecards.org/index.shtml.



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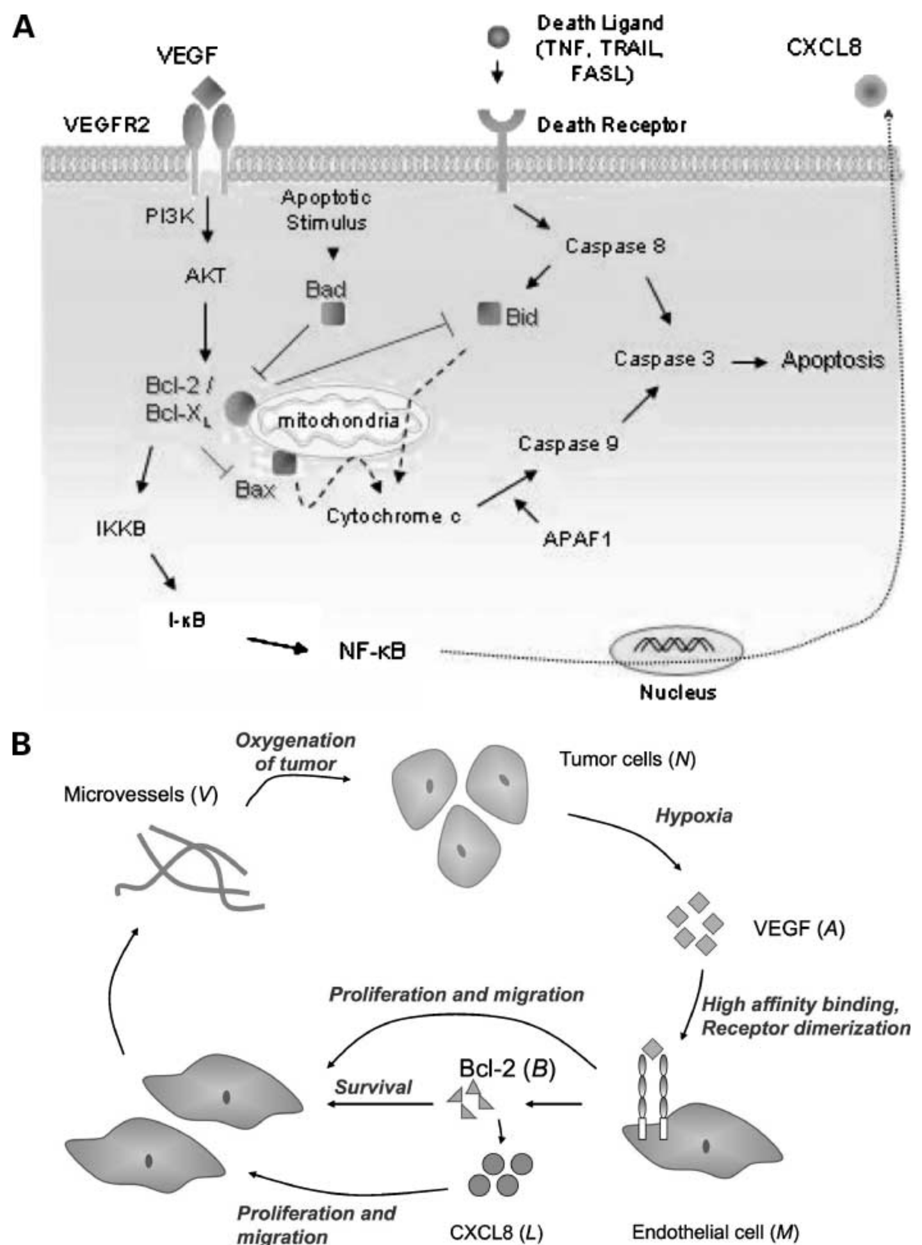


**Figure 1.** **A,** schematic diagram of intracellular functions of the Bcl family of proteins. VEGF induces Bcl-2 expression via the VEGFR2, PI3K/Akt signaling pathway. Proapoptotic proteins such as Bad and Bid heterodimerize with Bcl-2/Bcl-$X_L$; thus, regulating their ability to inhibit the activation of other proapoptotic proteins like Bax. Activation of Bax results in the release of cytochrome *c* from the mitochondrial outer membrane, which, together with Apaf1, causes caspase activation. This induces cell apoptosis. Bcl-2 also acts as a proangiogenic signaling molecule by activating the nuclear factor κB signaling pathway, inducing expression of the proangiogenic chemokine, CXCL8. **B,** a partial model schematic diagram. Tumor cells produce VEGF under conditions of hypoxia, which binds to endothelial cells via cell surface receptors resulting in receptor dimerization and activation. This elicits a proliferative and chemotactic response from the endothelial cells. Furthermore, this causes overexpression of the prosurvival protein Bcl-2, which in turn, results in the upregulation of CXCL8 production. CXCL8 in turn induces cell proliferation and chemotaxis. The endothelial cells begin to aggregate and differentiate into microvessels that eventually fuse with mouse vessels and become blood borne, resulting in oxygenation of the tumor.

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to the presence of cytochrome *c* in the cell cytoplasm, which together with Apaf1, activates caspase 9. This in turn activates downstream effector caspases such as caspase 3, which induce apoptosis (10). The Bcl family of proteins helps regulate this process by controlling the release of cytochrome *c*, typically from the mitochondrial outer membrane. Broadly speaking, the proapoptotic members of the Bcl family may be divided into two subfamilies. Members of the Bax-like subfamily include Bax and Bak, and are very similar to Bcl-2 in sequence, whereas the BH3-only proteins including Bad and Bid bear no sequence similarity to the members of the Bcl family apart from containing a BH3-binding domain (10). Members of the BH3-only subfamily bind to BH3-binding pockets that form on the antiapoptotic proteins such as Bcl-2 and Bcl-$X_L$, preventing them from inhibiting the activation of members of the Bax-like subfamily. It is believed that this results in the release of cytochrome *c* into the cell cytoplasm (3, 10). Thus, the antiapoptotic function of Bcl-2 and Bcl-$X_L$ is at least partly due to their ability to heterodimerize with Bax, Bad, Bak, and Bid, inhibiting their proapoptotic function (3). The various pathways that Bcl-2 and its family are involved in, are shown in Fig. 1A.

The Bcl family proteins have been shown to play a key role in tumor development and progression. Tumor cells in several types of cancers such as prostrate, breast, colorectal, head and neck cancers, lymphomas, and melanoma are

known to overexpress either Bcl-2 or Bcl-$X_L$, or both (3). In addition to promoting cell survival, this could provide cancer cells with some measure of protection from chemotherapy and radiotherapy, especially if these therapies directly or indirectly induce apoptosis (3). Furthermore, Nör et al. have shown that in the case of some head and neck cancers, cancer cells are able to extend this protective effect to the endothelial cells lining the blood vessels in the vicinity of the tumor by upregulating levels of Bcl-2 within these cells, thus enhancing intratumoral angiogenesis (11–13). This makes Bcl-2 and Bcl-$X_L$ attractive targets for the development of anticancer drugs. In fact, several forms of therapy targeting Bcl-2/Bcl-$X_L$ are under development. These include antisense bcl-2 and Bcl-$X_L$ oligonucleotides that work by inhibiting Bcl-2/Bcl-$X_L$ expression levels, single chain antibodies and peptides that bind to the Bcl-2 molecule inhibiting its functions, and an anti–Bcl-2 ribozyme that works by degrading bcl-2 mRNA (3). However, Wang et al. (3) propose that nonpeptidic, cell-permeable small molecule inhibitors of Bcl-2 and Bcl-$X_L$ may have greater potential as anticancer drugs than the therapies mentioned above for a variety of reasons including better bioavailability, stability, low cost, and the ability to penetrate the blood-brain barrier of the central nervous system. These inhibitor molecules act as antagonists of Bcl-2/Bcl-$X_L$ by binding to their BH3 binding pocket, thus preventing proapoptotic members such as Bax, Bad, and Bak from binding to Bcl-2/Bcl-$X_L$.

With all of these new possibilities, a critical challenge in experimental therapeutics for cancer is to decide which drugs are the best candidates for clinical trials. Mathematical modeling, such as that developed here can help to determine which anticancer drugs have the most potential for therapeutic benefit. In particular, we concentrate on the antiangiogenic potential of therapies targeted at Bcl-2 in the form of small molecule inhibitors such as BL193 (14, 15) and TW37 (15); both of which have been shown to cause a marked decrease in angiogenic potential of endothelial cells *in vitro*. Because BL193 has similar inhibition constants for both Bcl-2 and Bcl-$X_L$, we focus on this as our drug of choice. Our model is based on the experiments of Nör et al. (11–13), wherein human dermal microvascular endothelial cells (HDMEC) along with oral squamous carcinoma cells are transplanted into severe combined immunodeficient mice on biodegradable polymer scaffolds. These endothelial cells differentiate into functional human microvessels that anastomose with neighboring mouse vessels, thus generating human tumors vascularized with human microvessels (13). It has been observed that the proangiogenic chemokine vascular endothelial growth factor (VEGF) is the main tumor growth factor secreted in these experimental systems. VEGF mediates a proliferative and migratory response from the HDMECs, and exerts a prosurvival influence by upregulating intracellular Bcl-2 levels (11, 12). Bcl-2 in turn causes the endothelial cells to increase the production of interleukin-8 (CXCL8), which is a strong chemotactic agent and a potent inducer of endothelial cell mitosis (11). We have already developed and presented a mathematical model describing these experiments (16), and it is our goal to extend this model to explicitly incorporate the proapoptotic as well as antiapoptotic members of the Bcl family, so that we can better understand the implications and effects of therapy targeted against these proteins.

Our preliminary mathematical model (16) was the first to connect the molecular events associated with VEGFR2 dimerization and intracellular signaling with the temporal changes in endothelial cell proliferation, migration, and survival. We were able to use this model to predict the effect of decreasing the bioavailability of VEGF, CXCL8, and Bcl-2 on tumor growth and vascular structure. Our preliminary results suggest that Bcl-2 is the most promising target for antiangiogenic therapies along the VEGF pathway of interest. These results also led to the hypotheses that anti–Bcl-2 therapies applied at early and late stages of tumor growth will significantly affect, with a dose-dependent threshold, both the time course of tumor development as well as the maximum tumor cell and blood vessel densities. In this article, we will test this hypothesis by explicitly modeling the cellular response to variations in proapoptotic and antiapoptotic proteins in the Bcl family, as well as the administration of specific antiangiogenic therapies targeted against the VEGF-Bcl-2-CXCL8 pathway at different stages of tumor development. A partial model schematic is shown in Fig. 1B.

Due to the abundance of experimental data describing the interaction of Bcl-2 with its proapoptotic family members (1, 2, 4, 10, 17), and the increasing interest in the mechanism of action and therapeutic potential of the small molecule inhibitor BL193 (3, 14), it is important to develop a mathematical approach that includes the current understanding of the intracellular apoptotic signaling which is mediated by the Bcl family of proteins. Although our preliminary model could not address the mechanism of action of BL193 and could not relate its binding efficiency to its therapeutic efficacy, the model presented here will be able to study both of these

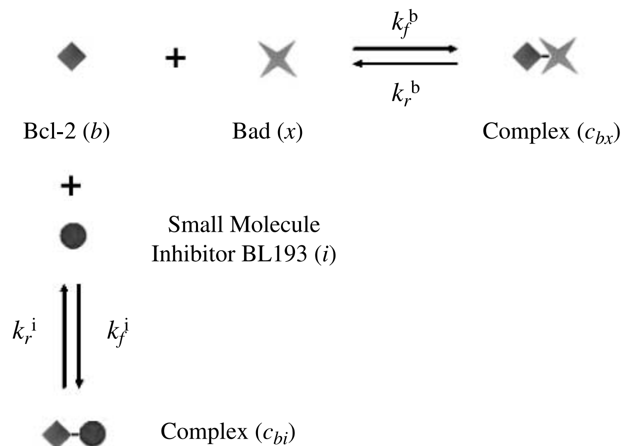


**Figure 2.** Reaction diagram of the heterodimerization reaction between the Bcl-2 (*b*) and Bad (*x*) molecules, and the inhibition of Bcl-2 by a small molecule inhibitor, BL193 (*i*).



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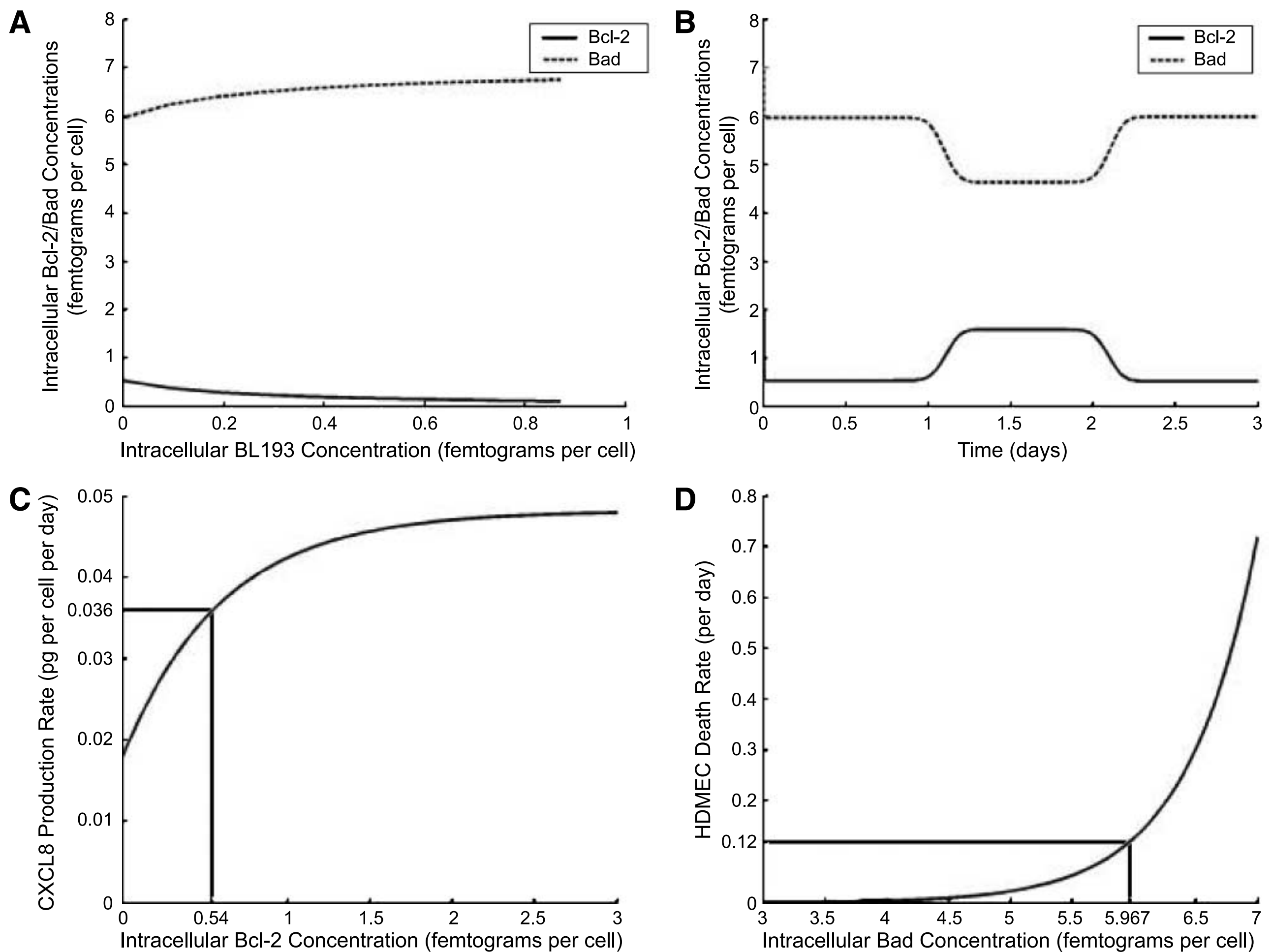


**Figure 3.** Intracellular Bcl-2 and Bad concentrations, and their effect on cell death rate and CXCL8 production rate. **A,** Bcl-2 levels within a single cell are seen to decline as increasing amounts of BL193 therapy are administered, causing a corresponding increase in Bad levels. **B,** upon application of 50 ng/mL of VEGF, Bcl-2 levels within cells are seen to increase 3.1-fold, causing a 22% decrease in the levels of Bad. As VEGF is consumed, Bcl-2 and Bad protein levels return to their constitutive state. **C,** CXCL8 production rate by HDMECs increases to a maximum as intracellular Bcl-2 levels increase. At the constitutive level of Bcl-2, CXCL8 production rate is fixed at 0.0358 pg of CXCL8 per HDMEC per day (16). The model allows for CXCL8 production independent of Bcl-2 upregulation as well. **D,** HDMEC death rate is taken to increase exponentially with the amount of intracellular Bad protein. At the constitutive level of Bad, cell death rate is taken to be 0.12/d (21).

important issues as well as predict tumor response with increased accuracy.

## Materials and Methods

### Model Foundation

A biochemically motivated, ordinary differential equation model was developed to capture the essential intracellular dynamics of the Bcl family of proteins governing programmed cell death. The mathematical model has as its foundation specific biological assumptions that are based on the accepted knowledge of the function of and cellular response to various proapoptotic and antiapoptotic proteins. Specifically, the intracellular concentration, in femtograms per cell, of a single antiapoptotic protein Bcl-2, and a single proapoptotic representative of the Bcl family, Bad, will be tracked in time. Using one representative of the proapoptotic and antiapoptotic family members allows us to avoid involving a number of intracellular binding parameters for which there are no experimental data. The particular choice of the representative proteins was made for the following reasons: (*a*) BL193 has similar binding affinities to both of the antiapoptotic proteins, Bcl-2 and Bcl-$X_L$, on which it has been shown to act (15); (*b*) it is the intracellular levels of Bcl-2 that are upregulated in response to a VEGF stimulus (12); and (*c*) Bad binds to Bcl-2 with a higher affinity than the other Bcl proteins (3). It also acts upstream of the Bax-like members of the Bcl family, making it the protein of choice to represent the proapoptotic members of the Bcl family. The intracellular concentration of Bcl-2 will be represented by the letter $B$, whereas that of Bad will be represented by the letter $X$.

It is known that Bcl-2 and Bad interact with each other within the endothelial cell to form heterodimers. This



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balance between proapoptotic and antiapoptotic proteins regulates cell death rate. In an endothelial cell, unbound Bcl-2, unbound Bad, and Bcl-2-Bad dimers will be present at their respective constitutive levels. We assume that the amount of unbound protein Bad determines the cell death rate because it is the proapoptotic members of the Bcl family that are directly responsible for regulating caspase activation within the cells (10). It has experimentally been shown that Bcl-2 induces the expression of CXCL8 in endothelial cells through its ability to activate the nuclear factor κB signaling pathway (14). Therefore, we assume that the amount of unbound Bcl-2 is responsible for CXCL8 production by the endothelial cells. The VEGF-Bcl-2-CXCL8 pathway discovered in the experiments of Nör et al. (11, 12) is explicitly included in this model by allowing for the upregulation of Bcl-2 in the presence of VEGF. In turn, this upregulates the production of CXCL8 by the endothelial cell due to increase in free Bcl-2 levels within the cell. High levels of Bcl-2 would mean low levels of Bad in its unbound state because it would be taken up as Bcl-2-Bad dimers. This would result in a decrease in cell death rate. Thus, we are able to capture the essential principles of cell death regulation by the Bcl family proteins, as well as account for the prosurvival and proangiogenic effect of Bcl-2 in particular. The complete derivation of equations is discussed in the sections that follow.

### Bcl Protein Interactions Within a Single Cell

We begin our model development at the single cell level, so that we may include the role of the Bcl family proteins in cell apoptosis explicitly. Once we are able to describe the relevant apoptotic pathways within a single cell, we can scale the model up to the population level, and thus obtain a better quantitative understanding of the role of these intracellular molecular pathways in determining vascular development in tumors.

**Bcl-2-Bad and Bcl-2-BL193.** The law of mass action is used to translate the reaction diagram in Fig. 2, which describes the heterodimerization of Bcl-2 and Bad as well as the inhibition of Bcl-2 by BL193, into a system of differential equations (Eqs. A–E) which govern the temporal changes in the intracellular concentrations of these proteins (18). Specifically, the uppercase letters in Eqs. A–E represent chemical concentrations, measured in femtograms per cell. Then, $B$ is unbound Bcl-2 protein per cell, $X$ is unbound Bad protein per cell, $C_{bx}$ is the quantity per cell of the heterodimer formed when one molecule of Bcl-2 binds to one molecule of Bad, $I$ is the amount of small molecule inhibitor or BL193 per cell, and $C_{bi}$ is the quantity per cell of the complex formed when one molecule of Bcl-2 binds to one molecule of BL193. Note that although we could find no experimental data on constitutive intracellular levels of Bcl-2 and Bad in HDMECs, there is evidence that Bcl-2, Bcl-XL, and Bax are expressed in femtogram levels in other cell types (19, 20).[5] To lend our model greater clinical applicability, the exact levels of intracellular Bcl-2 and Bad would need to be estimated experimentally. However, these numbers do not affect the qualitative predictions of our model.

It is assumed that upon application of therapy, the inhibitor molecules diffuse into the endothelial cell across the cell membrane. This is consistent with the design strategy behind these small molecule inhibitors (BL193 has a molecular weight of only 0.5 kDa; ref. 15). Thus, there is a source term in Eq. D, in which the rate of entry of the inhibitor molecules into an endothelial cell is proportional to the difference of extracellular and intracellular BL193 concentrations. The extracellular concentration, $I_0$, of BL193 in the local environment of a cell is a variable that varies as the dosage level of therapy changes, and is fit to match such experiments. The constant $D_i$ is a measure of cell wall permeability to BL193, and has units of 1/d. We can write the rate $D_i$ as $-(\ln 1/2)/t_{1/2}$, where $t_{1/2}$ is the amount of time it takes half the drug, external to a cell, to be internalized. The inhibition constant $K_i$ of BL193, which is given by the ratio of $k_r^i$ to $k_f^i$, is 320 nmol/L (15). The effect on intracellular Bcl-2 and Bad concentrations of the addition of varying amounts of BL193 can be seen from Fig. 3A. As BL193 levels within a cell increases from 0 to 0.9 fg, free Bcl-2 concentration is seen to decrease by 79%, causing free Bad concentrations to increase by 13% over their respective constitutive levels.

$$\frac{dB}{dt} = -k_f^b BX + k_r^b C_{bx} - k_f^i BI + k_r^i C_{bi} \qquad (A)$$

$$\frac{dX}{dt} = -k_f^b BX + k_r^b C_{bx} \qquad (B)$$

$$\frac{dC_{bx}}{dt} = -k_f^b BX - k_r^b C_{bx} \qquad (C)$$

$$\frac{dI}{dt} = -k_f^i BI + k_r^i C_{bi} + D_i(I_0 - \mathrm{I}) \qquad (D)$$

$$\frac{dC_{bi}}{dt} = k_f^i BI - k_r^i C_{bi} \qquad (E)$$

**The Effect of VEGF on Bcl-2 Levels.** Extracellular concentrations of cytokines and growth factors influence intracellular concentrations of the Bcl family proteins. In order to capture the effect of VEGF on Bcl-2 and Bad levels within each cell, we postulate that the amount of Bcl-2 produced is directly proportional to the number of active VEGFR dimer complexes per cell. A corresponding source term for

[5] Patent filed by Zychlinski et al., available online at http://www.patentgenius.com/patent/5972899.html.

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**Table 1. List of parameter values**

| Parameters | Value | Units | Source |
|---|---|---|---|
| $r_1$ | 1.2924 | per d | (16) |
| $C_1$ | 0.1 | Oxygen concentration | (16) |
| $r_2$ | 0.001 | per Tumor cell density/d | (16) |
| $\sigma$ | 1.0029 | Dimensionless | (16) |
| $C_2$ | 0.054 | Oxygen concentration | (16) |
| $C_m$ | 0.2 | Oxygen concentration | (16) |
| $k$ | 16.0 | No. of microvessels/mm$^3$ | * |
| $\mu_a$ | 12.8810 | No. of HDMECs/pg of $D_a$/d | (11)† |
| $\mu_l$ | 806.9190 | No. of HDMECs/pg of $C_l$/d | (11)† |
| $a_d$ | $3.8780 \times 10^{-6}$ | per d | (11, 21)‡ |
| $b_d$ | 1.7329 | per Bad concentration | (11, 21)‡ |
| $\alpha_1$ | 30.0 | No. of HDMECs/microvessel | (16) |
| $M_0$ | $17 \times 10^3$ | No. of HDMECs/mm$^3$ | (16) |
| $\alpha_2$ | 0.2984 | No. of microvessels/pg of $D_a$/d | (22)† |
| $\alpha_3$ | 6.5757 | No. of microvessels/pg of $C_l$/d | (22)† |
| $\eta_1^a$ | 0.2250 | pg VEGF/pg $R_a$ | (16) |
| $\eta_2^a$ | 0.1837 | pg VEGF/pg $C_a$ | (16) |
| $\eta_3^a$ | 0.8163 | pg $R_a$/pg $C_a$ | (16) |
| $\eta_4^a$ | 0.4494 | pg $R_a$/pg $D_a$ | (16) |
| $\eta_5^a$ | 1.2250 | pg $C_a$/pg $R_a$ | (16) |
| $\eta_6^a$ | 0.5506 | pg $C_a$/pg $D_a$ | (16) |
| $\eta_7^a$ | 2.2250 | pg $D_a$/pg $R_a$ | (16) |
| $\lambda_a$ | 15.5958 | per d | (16) |
| $r_3$ | 0.1507 | pg VEGF/tumor cell/d | (12)§ |
| $k_{f1}^a$ | 1.6232 | per VEGF concentration/d | (16) |
| $k_{r1}^a$ | 49.3025 | per d | (16) |
| $k_{f2}^a$ | 162.32 | per $C_a$ concentration/d | (16) |
| $k_{r2}^a$ | 0.493025 | per d | (16) |
| $k_p^a$ | 16.0 | per d | (16) |
| $V_{\mathrm{char}}$ | 55.0 | No. of microvessels/mm$^3$ | (16) |
| $V_0$ | 2.0 | No. of microvessels/mm$^3$ | (16) |
| $\epsilon$ | 1.0 | No. of microvessels/mm$^3$ | (16) |
| $k_f^l$ | 6.7587 | CXCL8 concentration/d | (16) |
| $k_r^l$ | 43.2557 | d | (16) |
| $k_p^l$ | 24.0 | d | (16) |
| $\eta_1^l$ | 0.1311 | pg CXCL8/pg $R_l$ | (16) |
| $\eta_2^l$ | 0.1159 | pg CXCL8/pg $C_l$ | (16) |
| $\eta_3^l$ | 0.8841 | pg $R_l$/pg $C_l$ | (16) |
| $\eta_4^l$ | 1.1311 | pg $C_l$/pg $R_l$ | (16) |
| $\lambda^1$ | 15.5958 | per d | (16) |
| $\beta_m$ | $8.1139 \times 10^{-4}$ | pg of CXCL8/HDMEC/d | (11, 16)†,‖ |
| $a_p$ | $6.9620 \times 10^{-4}$ | pg of CXCL8/HDMEC/d | (11, 16)†,‖ |
| $b_p$ | 1.6185 | per Bcl-2 concentration | (11, 16)†,‖ |
| $\alpha_4$ | 0.24845 | No. of microvessels/HDMEC | (16) |
| $k_f^b$ | 4 | per Bad concentration/d | * |
| $k_r^b$ | 8.832 | per d | (3) |
| $k_f^i$ | 40 | per BL193 concentration/d | (3)¶ |
| $k_r^i$ | 2.56 | per d | (3)¶ |
| $\beta_a$ | 40 | fg of Bcl-2/pg$D_a$ | (12) |
| $D_i$ | 3.4650 | per d | * |

*In the absence of experimental data, biologically realistic values for these variables were chosen so that the solution profiles best fit the experimental observations.

†The variables associated with CXCL8 production, and CXCL8 and VEGF effect on HDMEC proliferation and microvessel formation were estimated using least-square fits of experimental data in refs. (11, 22).

‡We know that the normal death rate of HDMECs is 0.12/d (21). Furthermore, in the presence of 50 ng/mL of VEGF, Bcl-2 is upregulated 3.1-fold, and consequently, the level of Bad decreases by a factor of 0.77. We assume a very low HDMEC death rate corresponding to this low level of Bad. From these two observations, the constants $a_d$ and $b_d$ may be determined.

§Keeping all other parameter values fixed, VEGF production rate by tumor cells was determined by fitting the microvessel density after 21 d with that observed experimentally in a (control) tumor that was allowed to grow without the application of any anticancer therapy (12).

‖A relation between the constants $\beta_m$, $a_p$, and $b_p$ may be found by observing that at constitutive Bcl-2 levels, the production rate of CXCL8 is 0.0358 pg of CXCL8 per HDMEC per day (16). Note that at constitutive levels of Bcl-2 and Bad, the cell death rate is taken to be 0.12/d (21) and HDMEC CXCL8 production rate is fit to experiments in ref. (12).

¶It was assumed that rate of forward reaction of BL193 with Bcl-2 was higher than that of Bad binding to Bcl-2 due to the much smaller size of BL193.

Bcl-2 may be included in Eq. A, which changes to Eq. F given below.

$$\frac{dB}{dt} = -k_f^b BX + k_r^b C_{bx} - k_f^i BI + k_r^i C_{bi} + \beta_a \frac{d}{dt}(\varphi_a) \qquad (F)$$

Here, $\phi_a$ is the density of active VEGFR dimer complexes in picograms per cell. In the presence of 50 ng/mL of VEGF, it is observed that Bcl-2 is upregulated by 3.1-fold (12). This is used to fix the constant $\beta_a$, which determines the level of upregulation of Bcl-2 by VEGF. In Fig. 3B, levels of Bcl-2 and Bad within a single cell are plotted versus time. An external dose of 50 ng/mL of VEGF is given and lasts for 1 d. It can be observed that unbound Bcl-2 concentration increases by 3.1-fold as VEGF is taken up by the cell. Corresponding to this, unbound Bad concentration decreases. As the VEGF is consumed, the amounts of Bcl-2 and Bad proteins eventually fall back to constitutive levels.

The CXCL8 production rate of HDMECs (Supplementary Material; see Eq. 14), denoted by $\beta_l$, is taken to be dependent on intracellular Bcl-2 concentration, $B$. The functional form of $\beta_l(B)$ is taken as follows:

$$\beta_l(B) = \beta_m + a_p(1 - e^{-b_p B}) \qquad (G)$$

The CXCL8 production rate by HDMECs is assumed to increase to a maximum level, as intracellular Bcl-2 concentration increases, under the effect of VEGF. We also allow for the possibility that CXCL8 could be produced by

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endothelial cells independent of Bcl-2. Thus, if the intracellular Bcl-2 level decreases to zero, there is still a constant background rate of CXCL8 production, i.e., $\beta_m$. The remaining variables are determined by fits in the least squares sense to experimental data presented in ref. (11). Figure 3C shows a graph of the CXCL8 production rate. We can see that as intracellular Bcl-2 levels increase, which may be in response to the presence of VEGF, the production rate of CXCL8 also increases.

### Apoptotic Regulation at the Population Level

Temporal changes in Bcl-2 concentration, within a single cell, will now be explicitly incorporated into the population level model of tumor growth and sustained angiogenesis described in the Supplementary Material (Eq. 16). To accomplish this, the death rate of HDMECs, denoted by $\lambda_m$, is taken to be dependent on intracellular Bad concentration, $X$ (see Eq. 9; Supplementary Material). In this way, the behavior predicted by the single cell model described in the previous section is used to determine the response of a population of cells to their microenvironment. The functional form of $\lambda_m$ is taken as follows:

$$\lambda_m(X) = a_d e^{b_d X} \qquad (H)$$

In the absence of experiments designed to determine the precise functional dependence of cell death on proapoptotic members of the Bcl family, we choose an exponential form of the death rate. This reflects the fact that cells are highly sensitive to proapoptotic signals such as intracellular Bad levels, which are normally tightly controlled and balanced by antiapoptotic proteins such as Bcl-2. There is some indirect experimental justification for this, which can be seen from a comparison of the model predictions to *in vitro* therapy results, presented in the next section. Figure 3D shows a graph of HDMEC death rate as a function of its intracellular environment. The graph also gives insight into the effect of extracellular events on the fate of the cell. For instance, if as a result of anti–Bcl-2 therapy, 0.5 fg of BL193 is incorporated by an endothelial cell, then free Bcl-2 levels within the cell decreases by 66%, and free Bad levels increase by 11%, over their respective constitutive values (Fig. 3A). This causes an increase of 208% in the cell death rate.

The model described above cuts across multiple levels of biological organization, from intracellular signaling to tissue level tumor growth dynamics, providing greater insight into intratumoral vascular development. Specifically, in order to access the role of Bcl proteins on vascular tumor growth and treatment, the model described above (Eqs. A–H) is combined with a set of delay differential equations (see Supplementary Material) that keep track of temporal changes in tumor and endothelial cell densities, VEGF and CXCL8 concentrations and microvessel density. Additionally, intracellular concentrations of Bcl-2, Bad, and BL193 are also measured over time. These equations represent a mathematical translation of the model schematic presented in Fig. 1B, and the Bcl-2-Bad-BL193 interactions and their effect on cellular apoptosis as described in the preceding sections. The parameter values used in our model (Table 1), along with a brief note on their estimation are listed in Supplementary Material.

## Results

A series of numerical experiments using the model described in the previous sections are carried out to simulate both, microvessel formation *in vitro*, as well as tumor induced angiogenesis *in vivo*. To date, the effect of the small molecule inhibitor BL193 of Bcl-2, on sprout formation, has only been tested experimentally *in vitro* (14, 15). Therefore, we first validate our model by presenting a comparison of model simulations to these *in vitro* experiments. This is followed by *in vivo* simulations designed to predict the effects of the administration of BL193 on intratumoral angiogenesis. The dependence of drug efficacy on its 50% inhibition constant of Bcl-2, and on the permeability of the endothelial cell wall to the drug are studied in particular. These are both key factors in drug design strategy. Therefore, the results of our simulations may be used to guide *in vivo* experimentation.

### *In vitro* Therapy

In a series of *in vitro* experiments described in ref. (14), capillary sprouting assays were carried out on HDMECs. The endothelial cells were exposed to 50 ng/mL of VEGF for 5 days and then to 50 ng/mL of VEGF in the presence of 0 to 5 μmol/L of BL193 thereafter, and the number of sprouts counted at daily intervals. To represent this experimental system, our model of intratumoral angiogenesis needs to be modified slightly by removing the tumor cell equation, and keeping free VEGF concentrations fixed at 50 ng/mL. The resulting system of equations is listed in the Supplementary Material. The values of all variables barring the amount of drug administered $I_0$, were estimated prior to the simulation of treatment strategies, and these values were kept constant thereafter. This allows us to validate our model by direct comparison of model predictions of treatment by BL193, with experimental observations.

Numerical and experimental observations are compared in Fig. 4A, B and C, in which the dosage of BL193 administered starting from day 5 increases from 0.05 to 0.5 μmol/L, and finally to 5 μmol/L. The model predictions of microvessel densities are seen to match the experimental observations well, thus validating our model. It should be noted that at this final concentration of BL193, the cells simply die from too much chemical within them, rather than from a disruption in the Bcl-2–regulated antiapoptotic pathway. The model does not account for such a possibility, and the last data point in Fig. 4C therefore does not match *in vitro* observations. The effect of therapy on intracellular protein levels can be seen in Fig. 4D, which plots the amounts of antiapoptotic protein B, proapoptotic protein X, and BL193 per cell, corresponding to a therapy level of 0.05 μmol/L of BL193.

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### *In vivo* Therapy

Having validated our model by comparison to *in vitro* experimental results, we can now perform a series of numerical simulations to investigate the effect of BL193 therapy on *in vivo* tumor growth dynamics when applied at an early stage of tumor development.

First, a series of numerical simulations were done to investigate the effect of therapy on tumor growth dynamics when applied at an early stage of tumor development. Prior to treatment, with variables at their baseline values, the tumor cell density reaches its maximum level ($8.669 \times 10^3$ cells/$mm^3$) approximately 28 days after implantation (Fig. 5A). The first blood-bearing vessels are seen 5 days after implantation, reaching their steady state of ~53 vessels/$mm^3$ 19 days later (Fig. 5B). When a dose of 0.05 μmol/L of BL193 is administered, it takes about a week longer for the vasculature to fully develop, and the tumor cell density to reach its maximal value, when compared with the no therapy case. If the dosage is increased to 0.5 μmol/L, the delay in vascular development increases by 16 days, whereas the tumor cells take 18 days longer to reach their steady state. A dosage level of 5 μmol/L of BL193 seems to be enough to cause the tumor to regress (Fig. 5A and B). Thus, based on these numerical predictions, antiangiogenic therapies directed at Bcl-2, such as the use of small molecule inhibitors of Bcl-2, seem to be highly efficacious, especially when administered to an early stage tumor.

Next, we investigate the effect of application of BL193 to a tumor that has been allowed to reach maximal tumor cell and microvessel densities (Fig. 5C and D). Therapy is applied continuously from day 60 of implantation. The model predicts that at a dosage level of 5 μmol/L BL193, the vessel density is seen to decrease (Fig. 5D), leading to a corresponding decrease in tumor cell density (Fig. 5C). However, the tumor cells seem to compensate for this loss of blood supply by increasing VEGF production. The vessel

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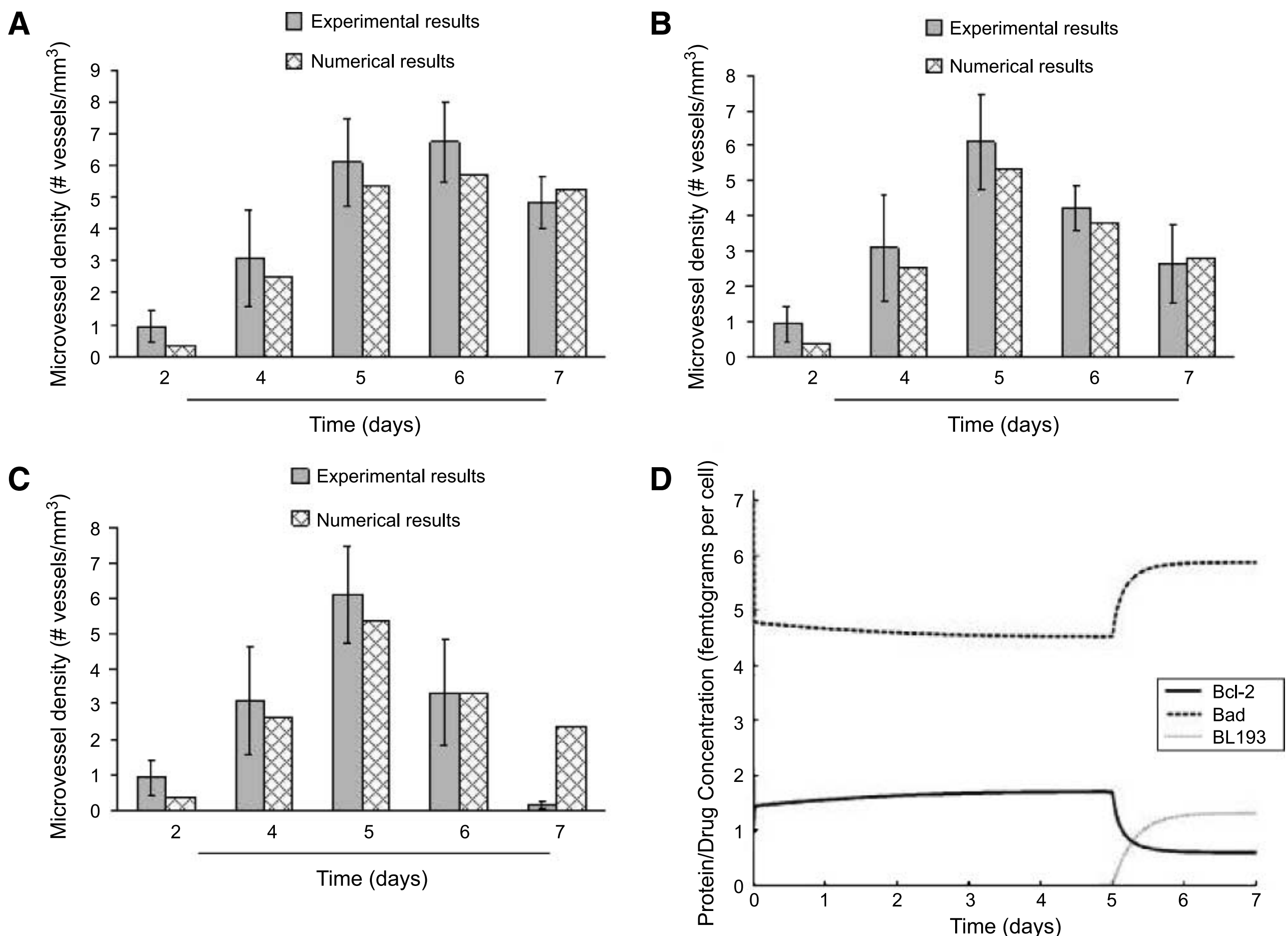


**Figure 4.** Comparison of model simulations to *in vitro* simulations designed to study the effect of BL193 on capillary formation. **A** to **C**, in capillary sprouting assays described in (14), HDMECs were cultured on type I collagen in the presence of 50 ng/mL of VEGF. Starting on day 5, BL193 was administered in increasing doses from 0.05 (**A**), 0.5 (**B**), and 5 μmol/L (**C**), and the number of sprouts counted. Numerical simulations of our model are seen to be in good agreement with experimental data, thus validating it. **D**, intracellular Bcl-2, Bad, and BL193 levels are tracked with time, as 0.05 μmol/L of BL193 therapy is administered. Starting on day 5, BL193 levels within a cell begin to increase, causing a decrease in amounts of unbound Bcl-2 protein and a corresponding increase in unbound Bad protein.

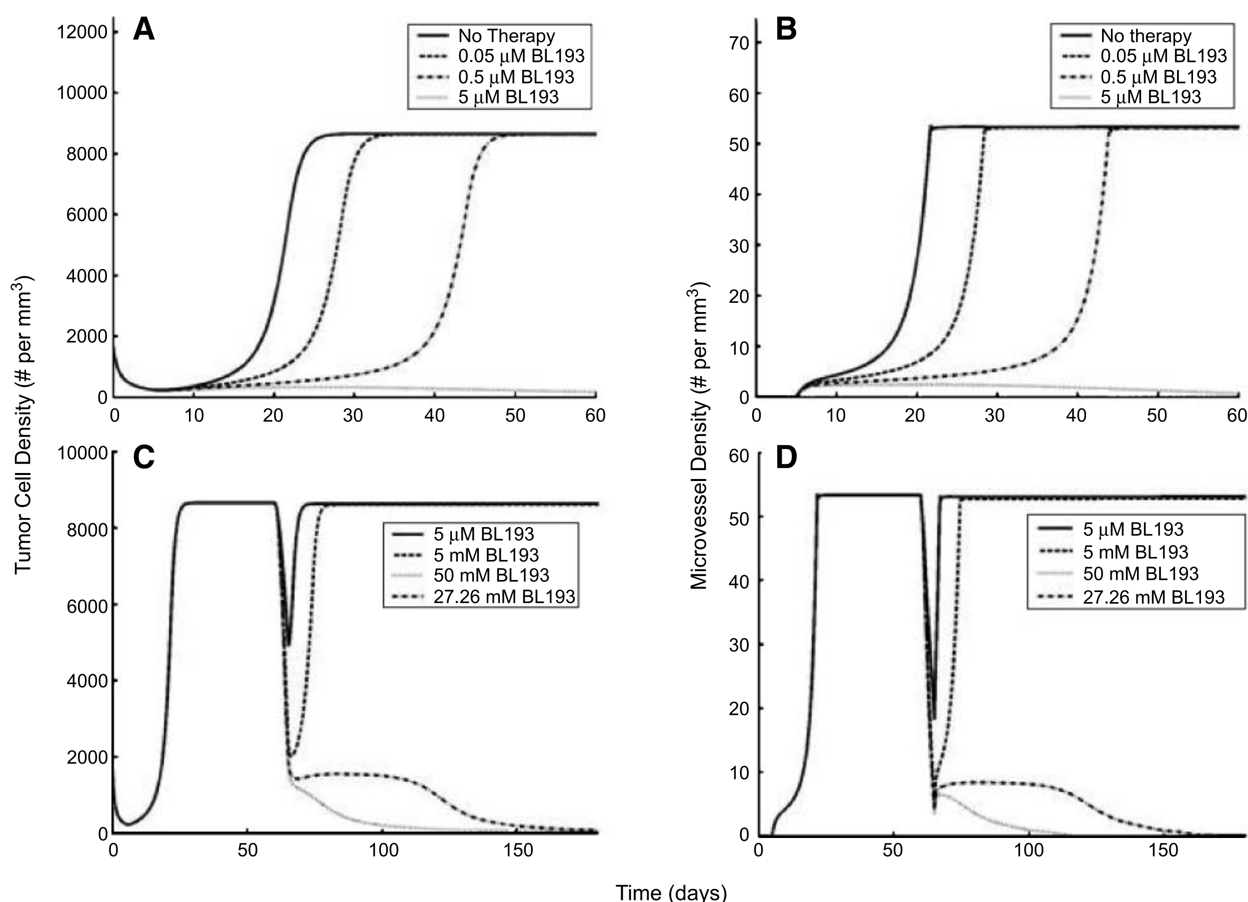


**Figure 5.** *In vivo* simulations of anti–Bcl-2 therapy applied to a tumor at early and late stages of development. Our model is based on experiments described in (11–13), wherein HDMECs, along with oral squamous carcinoma cells, are transplanted into severe combined immunodeficient mice on poly-L lactic acid matrices. The HDMECs are observed to differentiate into functional microvessels, giving rise to a vascularized tumor. **A** and **B,** BL193 is administered starting from the day of implantation and continuing thereafter. As therapy levels increase from 0 to 0.05 μmol/L, and then to 0.5 μmol/L, the time taken to reach the maximal tumor cell density increases by 25% and 89%, respectively (**A**). The corresponding increase in time taken to reach maximal vessel density was 37% and 121%, respectively (**B**). Five microliters of BL193 seems to be enough to effect a cure. **C** and **D,** BL193 is administered to a fully developed tumor, starting from day 60 of implantation and continuing thereafter. Five microliters of BL193 was insufficient to effect a cure, and only a temporary reduction in tumor cell (**C**) and vessel densities (**D**) was observed. The minimum amount of therapy required in order to cause tumor regression is predicted to be 27.26 mmol/L.

density soon returns to its pretreatment level, and the tumor recovers rapidly. In fact, we need to increase the dosage of BL193 to ~24.5 mmol/L to induce tumor regression. In this case, the microvessel density is observed to decrease to a level that is too low for the tumor to recover from as a result of anti–Bcl-2 therapy (Fig. 5D).

### Sensitivity to Drug Design Parameters

There are two important considerations behind the design strategy of small molecule inhibitors of Bcl-2, such as BL193. First, the need to maximize the inhibition of Bcl-2 by the molecule. Second, the need to maximize the cell-permeability of the molecule. We therefore carry out a sensitivity analysis on the inhibition constant $K_{Di}$ of BL193, and the rate of diffusion $D_i$ of BL193 across the cell wall. In all the simulations carried out here, the drug is administered to a late stage tumor, and the least amount of drug required to induce tumor regression is observed. Numerical simulations predict that decreasing $K_{Di}$ exponentially decreases the amount of drug required to effect a cure (Fig. 6A). For instance, the required dosage of an inhibitor with a $K_{Di}$ 25% lower than that of BL193 is 0.52 mmol/L, whereas that of an inhibitor with a $K_{Di}$ 50% lower than that of BL193 is 11.04 μmol/L. The required dosage for BL193 is 27.26 mmol/L. Likewise, increasing the cell-permeability of BL193, i.e., increasing the rate of diffusion of BL193 across the cell wall, reduces the level of therapy required (Fig. 6B). A 10-fold increase in $D_i$ reduces the minimum drug dosage needed for a cure by a factor of 53%, but any increase in $D_i$ beyond this does not significantly affect this dosage further.

## Discussion

A deeper understanding of the cellular and molecular events that govern tumor-induced blood vessel growth is crucial to the development of anticancer therapies targeted at angiogenesis. Therefore, it has been the focus of intensive

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research, and several experimental models have been developed to study it. One such experimental model investigated the importance of the upregulation of Bcl-2 by VEGF in sustained intratumoral angiogenesis (11, 12), and the effect of blocking this by means of a small molecule inhibitor BL193 of Bcl-2 (14). We previously published a mathematical model describing this experimental setup at an extracellular and tissue level (16). The main goal of this research was to incorporate the intracellular dynamics of the Bcl family of proteins in our existing model. This enabled us to quantify, in a more definitive way, the role Bcl-2 plays in intratumoral angiogenesis, and to assess its potential as a target for antiangiogenic therapy. By building a model specific to this experimental system, we were able to validate it by direct comparison with experimental results. The model was then used to simulate the application of therapy *in vivo*, thereby highlighting its usefulness as a guiding tool in the development of antiangiogenic drugs designed to inhibit the function of Bcl-2.

Numerical simulations of the full model provide valuable insight into the growth dynamics of tumors and their response to the application of BL193 therapy. The model was first calibrated versus control *in vitro* capillary sprouting assays. It was found to be in good agreement with experimental observations taken after the application of BL193. The model was then used to predict the effect of therapy applied at various stages of tumor growth *in vivo*. Numerical results indicated that even low levels of BL193, administered to an early stage tumor, induced significant delays in tumor and vascular development. In fact, 5 μmol/L of Bl193 was sufficient to cause the tumor to regress. This could be due to the fact that if the vasculature has not had a chance to develop in a tumor, then administering anti–Bcl-2 therapy has the effect of preventing further vascular development by inducing cell death. However, in a simulation carried out to test this drug on a fully developed tumor, we found that the amount of therapy required to effect a cure increased by three to four orders of magnitude. It was observed that below a certain threshold, anti–Bcl-2 therapy elicited little response from the tumor, but as therapy was increased beyond this threshold, the tumor and vascular development were arrested at much lower levels. However, it is possible that this threshold corresponds to an amount of therapy that is toxic for the host, and thus, not administrable in practice. These results accentuated the importance of timely detection and treatment of tumors, and indicated that a single point of attack at a fully developed tumor may be insufficient in order to effect a cure. Finally, numerical experiments were run to provide an insight into drug design strategies aimed at improving the effectiveness of the small molecule inhibitor therapy, especially in late stage tumors. A small molecule that was only 25% more efficient than BL193 in inhibiting Bcl-2 reduced the required therapy level by as much as 97%. Therefore, even a moderately better inhibitor of Bcl-2 may prove to be a much better target for the development of antiangiogenic therapy. However, increasing the cell wall permeability of the drug molecules could reduce the minimum effective therapy level by at most 56%. These numerical simulations suggest that improving drug design in terms of permeability across cell walls has limited potential as far as minimizing drug efficacy levels is concerned. Continued quantitative modeling in this direction could have a profound effect on the development of antiangiogenic drugs aimed at the intracellular regulators of cell death.

The research carried out in this article transcends the boundaries between biomedicine and mathematics, and has the potential to aid efficient drug design and to optimize administration protocols. This type of modeling has proved useful to experimentalists by providing a quantitative rationale

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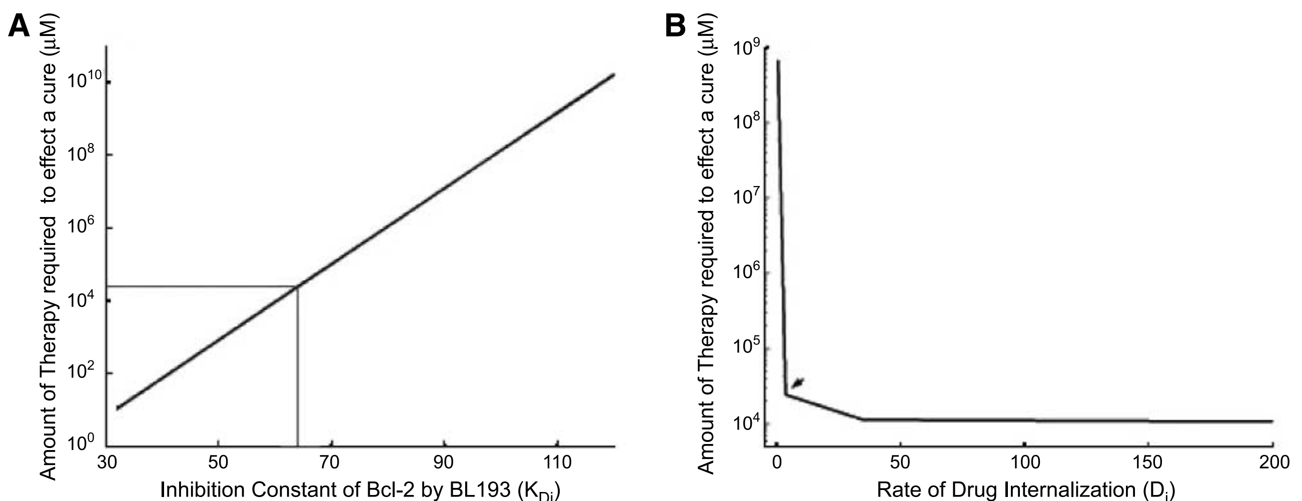


**Figure 6.** Simulations to guide drug design strategies for anti–Bcl-2 therapy. **A,** minimum amount of therapy required to induce tumor regression is observed to vary exponentially with the inhibition constant $K_{Di}$ of BL193 for Bcl-2. Reducing $K_{Di}$ from its baseline value by 25% decreases the least amount of therapy required from 27.26 to 0.52 mmol/L. **B,** as the rate of diffusion $D_i$ of BL193 into the cell is increased, the least amount of efficacious therapy reduces by a maximum amount of 56%, after which no significant change is observed when compared with baseline values for $D_i$.

for anti–Bcl-2 therapy as the more effective target for the development of an antiangiogenic drug. However, eventual clinical application of theoretical modeling such as that carried out here necessitates the accurate estimation of a number of variables in a defined experimental setting. Building such a data set is therefore of paramount importance. This underscores the need for a close collaboration between researchers from biomedicine, mathematics, and physics in order to advance our understanding of diseases such as cancer and arrive at effective treatment strategies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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