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# Targeting cholesterol with β-cyclodextrin sensitizes cancer cells for apoptosis

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ABSTRACT

**We found that targeting cholesterol with beta-cyclodextrin (bCD) and its derivatives disrupted signal transduction between PI3K and AKT, attenuating AKT pro-survival signals. In their absence, 2-deoxyglucose (2DG) caused anti-apoptotic protein Mcll to dissociate from pro-apoptotic Bak at mitochondria. Normally Bak is sequestered by its inhibitory associations with Mcll and Bcl-xL, and only when Bak is released from both, is it free to form oligomers through which cytochrome *c* can escape into the cytosol. Thus an addition of a bcl-2 antagonist dissociates Bak from Bcl-xL, triggering cytochrome *c* release and inducing apoptosis. 2DG–bCD can also sensitize type II cancer cells for TRAIL-mediated apoptosis.**


## 1. Introduction

A single cancer cell left behind after surgery and/or chemotherapy could cause the recurrence of cancer [1]. Therefore, the aim of cancer chemotherapy must be to eliminate all cancer cells. Given the heterogeneity of cancer cells in a tumor, it is difficult to eliminate all cancer cells by a single agent targeting a particular gene product [2]. Thus, we developed a 2-deoxy glucose–ABT-263 (2DG–ABT) combination therapy [3]. This combination targets mitochondria directly, activating Bak to release cytochrome *c* into the cytosol where it forms the apoptosome, a protease complex that destroys numerous proteins. The apoptosome is often called the death executioner, and the release of cytochrome *c*, the point of no return. Thus, unlike other instigators of apoptosis, use of 2DG–ABT induces apoptosis very quickly, circumventing the many steps usually required for apoptosis induction, and reaches the point of no return within a few hours. The 2DG–ABT combination efficiently induced apoptosis in p53 compromised, PTEN deleted, highly chemo-resistant cancer cells [3]. As a single agent, neither 2DG nor ABT is very effective. However, when cells take up both agents, they synergize to induce apoptosis. 2DG targets highly glycolytic cells; in our body, these are cancer cells, cells in inflamed tissues, muscle cells under heavy exertion, and brain cells. In contrast, since ABT cannot cross the blood–brain barrier, it accumulates in all tissues outside the brain. Thus, by avoiding heavy

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exertion and limiting inflammation, the 2DG–ABT combination therapy can be made to target cancer cells outside the brain for apoptosis [2].

When 2DG–ABT was tested on tissue culture cells, it induced apoptosis in all types of cancer cells across varied genetic backgrounds. However, its efficiency varied from cell line to cell line. One reason for the varied efficacy is the varied strength of the phosphoinositide 3-kinase–AKT (PI3K–AKT) pro-survival signal present in cancer cells. Since the PI3K–AKT pathway is activated in many normal tissues, targeting this pathway for cancer therapy causes many adverse side effects. On the other hand, epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R) and other receptor tyrosine kinases (RTKs) are often activated in specific cancer types, leading to the activation of PI3K–AKT, and targeting a specific RTK with a specific inhibitor may also be an effective way to diminish the pro-survival signal in some cancer cells with fewer side effects [4]. For example, IGF1R is expressed in renal cancer cell lines RCC4 and U0K121, generating a PI3K–AKT pro-survival signal. A specific inhibitor of IGF1R such as picropodophyllin (PPP) could enhance the efficacy of 2DG–ABT combination therapies on these cells. However, a real tumor mass is not like cancer cells from a cell line; some cells in a tumor could express IGF1R while others may express EGFR or an insulin receptor in the same tumor, and they can all generate a PI3K–AKT pro-survival signal. Therefore, inhibiting just IGF1R or EGFR or even both would not be enough. For this reason, we tried inhibiting signals from all RTKs. To meet our goal, we used beta-cyclodextrin (bCD) and its derivatives. Administration of bCD in vitro and in vivo experiments diminished pro-survival signals for a few hours. When we applied the 2DG–ABT combination therapy during bCD-mediated suppression, cells went into apoptosis very efficiently in a manner consistent with mitochondria-dependent apoptosis. Treatment of mice carrying human cancer cells with 2DG–bCD–ABT combination caused tumor regression.

## 2. Results

### *2.1. bCD disrupted signal transduction between PI3K and AKT, interfering with RTK–PI3K–AKT pro-survival signals while leaving RTK–Ras–ERK proliferation signals intact*

Since HeLa cells express both EGFR and IGF1R, we tested the effects of bCD on EGF-stimulated and IGF 1-stimulated HeLa cells. To determine the concentration necessary for bCD to attenuate IGF 1-induced AKT activation, HeLa cells were first incubated in serum-free medium containing 0–7 mM bCD for 30 min before these cells were stimulated with 20 ng/ml IGF1 for 20 min. The results showed that 7 mM bCD was enough to completely block the IGF1 generated signal to AKT (Fig. 1A). Subsequently, we used 30 min pre-incubation with 10 mM bCD to alter RTK generated signal transduction pathways. To analyze the effects of bCD on RTK-generated pathways in more detail, we used HeLa cells cultured in serum-free medium stimulated with a combination of particular growth factors. Thus, HeLa cells were first incubated in serum-free medium with or without 10 mM bCD for 30 min before these cells were stimulated by 100 ng/ml EGF or 10 ng/ml IGF1 for the indicated durations. Both ligands activated their respective receptors as indicated by the phosphorylation of the receptors (Fig. 1B and C). Most RTKs activate two distinct signal transduction cascades: the RTK–Ras–ERK proliferation pathway and the RTK–PI3K–AKT pro-survival pathway. In untreated HeLa cells, both pathways were clearly activated, but in bCD-treated cells, even though both EGF and IGF1 activated PI3K, AKT activations were considerably diminished (the last three lanes in each of the bottom boxes). At the same time, RTK–Ras–ERK signals seem to be unaffected (the second boxes in Fig. 2B and C). Thus, bCD disrupted signal transduction between PI3K and AKT, diminishing PI3K–AKT pro-survival signals generated by these RTKs while leaving Ras–ERK proliferation signals intact. Lastly, while HeLa cells mostly express Class II PI3K, A431 epidermoid carcinoma cells express mostly Class IPI3K isoforms, and both their signals to AKT are attenuated by bCD (Figs. 1 and 3B). Thus bCD can interfere signal transduction from Class I and Class IIPI3K to AKT.

### *2.2. bCD and 2DG work synergistically to enhance ABT-induced apoptosis*

Since VHL-defective renal cancer cells such as RCC4 cells were less sensitive to 2DG–ABT largely because they express IGF1R [5], we wanted to see whether 2DG–ABT combined with bCD would increase its efficacy. However, we and others have also known that 2DG stimulates AKT phosphorylation in many cancer cell lines [3,6]. Thus, we first tested whether the dual treatment of 2DG with bCD would increase or decrease AKT phosphorylation in RCC4 cells. Since it generally takes 1–2 h for the effect of 2DG to become noticeable [3], whereas bCD works within 30 min (see Fig. 1), serum-staved RCC4 cells were first treated with 2DG for 2 h, and in the last 30 min, some cells were also treated with bCD. Yet other cells were treated with 2DG or bCD alone, or left untreated. Then all RCC4 cells were stimulated with 0–30 ng/ml IGF1 for 20 min. The results were very clear: even though 2DG pre-treatment sensitized these cells for IGF1, increasing the phosphorylation of AKT in 2DG treated cells, dual treatment with bCD completely blocked AKT phosphorylation (Fig. 2A). These results suggest that even though 2DG enhances IGF1R activities, signals from IGF1R did not reach AKT in the presence of bCD.

In order to test the effects of bCD on a particular RTK, in the above experiments, cells were incubated in serum-free medium and then stimulated with a particular growth factor. However, the serum used contains multiple growth factors as well as insulin that could activate multiple RTKs expressed in these cells. Thus, we needed to test whether bCD still modulates PI3K–AKT signals while they are continuously stimulated by serum. For this, we treated RCC4 cells with bCD, with 2DG and with their combination, and examined the phosphorylation status of AKT. Phosphorylation of AKT was almost totally absent in RCC4 cells kept in 10% serum while being incubated with bCD for 30 min and also in RCC4 cells kept in 10% serum while co-incubated with 2DG and bCD (Fig. 2B). However, it did not take long for AKT activities to come back when cells were returned to media without bCD but containing 10% serum (Fig. 2C). Several studies had shown that when one of the bCD derivatives, hydroxypropyl-β-cyclodextrin (HPBCD), is injected into mice, 4 h later only 50% of bCD would remain in circulation [7,8]. Thus, to take advantage of bCD-induced absence of pro-survival signals for cancer therapies, apoptosis needs to be induced fairly quickly. In order to test whether bCD affects PI3K–AKT pathways in animals, we developed our own assay in which 5-h starved mice were treated with 40 μg bCD 30 min before these mice were challenged with 100 ng IGF1, and observed blood glucose levels 30 min later. The mice all weighed approximately 20 g. The results are shown in Fig. 2D. bCD clearly attenuated IGF 1-induced hypoglycemia, suggesting that bCD partially blocked the signal transduction between IGF1R and AKT in animal bodies.

Because our aim is to develop cancer combination therapies that include bCD, we were pleased to learn that when introduced into animal bodies, bCD attenuates the IGF1R–PI3K–AKT pathway at a dose well within the safety limit established by the clinical trial for HPBCD for the treatment of Niemann–Pick disease [9]. On the other hand, bCD may not be retained in circulation at high enough doses to be effective for sufficient time. Taking into account these considerations, we developed the following in vitro protocol for combining bCD with 2DG–ABT so that it approximates

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