Adenosine A2A and Beta-2 Adrenergic Receptor Agonists: Novel Selective and Synergistic Multiple Myeloma Targets Discovered through Systematic Combination Screening

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Abstract

The use of combination drug regimens has dramatically improved the clinical outcome for multiple myeloma (MM) patients. However, to date, combination treatments have been limited to approved drugs and a small number of emerging agents. Using a systematic approach to identify synergistic drug combinations, combination high throughput screening (cHTS) technology, adenosine A2A and beta-2 adrenergic receptor agonists were shown to be highly synergistic, selective and novel agents that enhance glucocorticoid activity in B-cell malignancies. Unexpectedly, A2A and beta-2 adrenergic receptor agonists also synergize with melphalan, lenalidomide, bortezomib and doxorubicin. An analysis of agonists in combination with dexamethasone (Dex) or melphalan in 83 cell lines reveals substantial activity in MM and diffuse large B cell lymphoma cell lines. Combination effects are also observed with Dex as well as bortezomib, using MM patient samples and mouse MM xenograft assays. Our results provide compelling evidence in support of development of A2A and beta-2 adrenergic receptor agonists for use in multi-drug combination therapy for MM. Furthermore, use of cHTS for the discovery and evaluation of new targets and combination therapies has the potential to improve cancer treatment paradigms and patient outcomes.

Introduction

The identification of novel synergistic drug combinations is an important endeavor that undoubtedly will improve the outcome of cancer treatments. Unfortunately, to date, combination treatments have mainly been the result of using two (or more) agents already validated in the clinic or the pairing of approved with emerging drugs. As a result, the diversity of combination therapies is limited as synergistic drug pairings have been relatively few. Although there is clear evidence that cancer combinations can work better than monotherapies (1-3), to date, combination activities have not been systematically explored.

We developed a combination high throughput screening (cHTS) approach that allows us to explore the activity for thousands of compounds to identify paired
agents with synergistic activity (4,5). During screening, a dose matrix is generated that defines single agent and combination activity over a wide concentration range. Such a detailed analysis can help identify target-relevant effects and guide testing of lead drug combinations in vivo, as the dose matrix provides the concentration ratios where the drugs are synergistic and highlights the individual agent concentration ranges required to see combination activity. Our screening platform allows a deep exploration of drug combination space and is robust enough for analysis in many cell lines. The use of many cell lines is important, as knowledge about drug sensitivity/combination activities should be examined within the context of various genetic backgrounds and expression profiling to direct the use of drug combinations to patients most likely to respond to treatment.

Glucocorticoids are standard of care for a number of hematological malignancies including multiple myeloma (MM), non-Hodgkin’s lymphoma and acute lymphoblastic leukemia and are usually used in combination with other drugs for the treatment of these diseases. With MM, the first drug used in combination was the glucocorticoid (GC) prednisone, initially paired with melphalan (in 1969, ref 6). In the past decade, thalidomide, lenalidomide, bortezomib and doxorubicin have achieved FDA approval and along with melphalan, are frequently deployed as part of multi-combination therapies that include either prednisone or dexamethasone (7). Preclinical studies suggested these drugs could provide benefit in combination as enhanced activity (additive or synergistic) was observed for combinations vs. single agents (8-10). The successful translation of these preclinical studies points to the value of using current MM models for combination drug discovery.

MM.1S is a GC-sensitive MM cell line used frequently along with MM.1R, a derivative GC-insensitive line, to identify and characterize compounds that kill MM cells via GC-dependent and independent pathways (11). The issue of GC sensitivity is important as this class of drug is frequently less efficacious with long term use, requiring a switch to different drug regimens or use of additional drugs that might increase GC sensitivity. We have used the MM.1S cell line to identify two classes of drugs that synergize with dexamethasone (Dex) to kill MM cells. These agents are novel, have good breadth of activity for MM and possible utility for a wider variety of B-cell malignancies. We show that combination activity is
not restricted to GCs, the novel agents are synergistic with other standard of care drugs, suggesting benefit when used with currently deployed drug regimens.

Materials & Methods

Cell lines and Reagents

All cell lines were obtained from ATCC, DSMZ or Lonza and subject to their authentication methods. The exceptions are MM.1S (Dr. Stephen Rosen, Northwestern University) and SU-DHL-7, OCI-ly1, OCI-ly3, OCI-ly4, OCI-ly10 and OCI-ly19 (Dr. Margaret Shipp, Dana Farber Cancer Institute, Boston). Cell identity for non-commercial lines is supported by morphology, known drug sensitivities and gene expression studies. All compounds used in this study were obtained from commercial sources. See Figure S1 for information about MM drugs and the adenosine and beta-2 adrenergic receptor agonists used in this study.

Combination High Throughput Screening

Drug combination studies were performed by plating MM.1S cells in 384-well plates at 1000-1500 cells per well, cultured 18-24 hours and treated with increasing concentrations of each drug to generate a dose matrix (4,5). After 72 hours, cell viability was assessed by ATPlite luminescence using an EnVision 2103 according to the manufacturer’s instructions (PerkinElmer Life and Analytical Sciences, Inc, Waltham, MA).

Analysis of Combination Activity

A “dose matrix” was used to evaluate the combination effects between compounds, where all possible mixtures of serially-diluted single agents at the indicated concentrations were evaluated including zero (12-14). Synergy was determined by the Loewe additivity model, a generally accepted reference for synergy (15-17). The isobologram (18) shows how much less drug is required in combination to achieve a desired effect level, when compared to the single agent doses needed to reach that effect. The combination index (CI) is the ratio of the total effective drug dose of the combination compared to that of the single agents required to achieve a given effect level (16). A more detailed discussion of combination activity analysis can be found in the legend of Supplemental Figure 2.
Patient Samples

Samples collected from informed patients who had provided informed consent according to DFCI IRB-approved protocol. Tumor cells were isolated using CD138 microbeads (Miltenyi Biotec) and viability measurements performed as described (19).

Animal Studies

Male SCID mice 6-8 weeks of age (Charles River Labs) were used for MM.1S and H929 xenograft studies. Female SCID mice 5-6 weeks of age (Harlan, Inc.) were used for the RPMI-8226 studies. Animal shaven flanks were subcutaneously injected with 1.5 x 10⁷ logarithmically growing MM.1S and H929 cells or 1 x 10⁷ RPMI-8226 cells. Once tumors grew to 130-250 mm³ in size, animals were pair-matched by tumor size into treatment and control groups. Drug stocks were prepared in EtOH and diluted with PBS to generate a 10% EtOH:90% PBS solution for injections. Dex, CGS-21680 and salmeterol were delivered by subcutaneous (sc) injection daily while bortezomib was injected intravenously via the tail vein. Control animals received 10% EtOH:90% PBS sc daily. Animals were removed from the study if there was overall poor body condition, the tumor volume was above 3000 mm³, body weight loss was greater than or equal to 20% or if the tumors became ulcerated. Mean change in body weight and tumor volume was calculated on the last day when there was not more than one animal removed from each study group. Statistical differences were analyzed using an analysis of variance (ANOVA). Normal distribution was determined by the goodness of fit for normality test. When warranted by the omnibus test, a Tukey-Kramer HSD post hoc analysis was employed to determine differences between individual groups.

Microarray analysis

MM1.S cells were not treated or treated for 6 hours with CGS 21680 (12.5 nM), salmeterol (1nM), Dex (25nM or 2µM), CGS 21680 (12.5 nM) with Dex (25nM) or salmeterol (1nM) with Dex (25nM). Two biological replicates for each
Adenosine A2A and Beta-2 Adrenergic Receptor Agonists: Novel Selective and Synergistic Multiple Myeloma Targets Discovered through Systematic Combination Screening

treatment were used in the microarray study with Affymetrix U133 plus 2.0 chips. The data have been deposited in NCBI's Gene Expression Omnibus under accession number GSE30644 (20).

**Results**

*The identification of adenosine receptor and beta adrenergic receptor agonists as potent enhancers of multiple myeloma drugs*

The MM.1S MM cell line was used to screen approved drugs and molecular probes to identify targets that synergize with Dex to inhibit proliferation. A total of 1601 unique combinations were evaluated using a 384 well dose matrix format, which facilitates the identification of synergistic activity through analysis of drug combination activity over a wide concentration range (4,5). Two classes of compounds were found that are potently synergistic with Dex, exemplified by CGS-21680, an adenosine receptor (AdR) agonist (Figure 1A) and salmeterol (Figure 1E), a beta-2 adrenergic receptor (β2AR) agonist. Both agents, when combined with Dex, increase the maximal effect and potency of Dex (Figures 1B, 1F) and are strongly synergistic as revealed by isobologram analysis (Figures 1C, 1G) and the calculated excess inhibition over the predicted Loewe additivity model (Figure S2A). Other AdR agonists and β2AR agonists are also strongly synergistic in combination with Dex (Figures S2B and S2C) suggesting that activation of these receptors is important for activity. As combination activity is observed broadly in many MM cell lines (Figures S3A, S3B and see below) AdR and β2AR agonists may represent novel MM drug targets.

GCs are invariably used in the clinic in combination with one or more drugs. As AdR or β2AR agonists might be added to existing MM combination therapies, understanding how these agonists affect the activities of other approved MM drugs is important. We therefore examined the anti-proliferative activity of melphalan, lenalidomide, bortezomib and doxorubicin in combination with either an AdR agonist or β2AR agonist using the MM.1S cell line. Surprisingly, synergy was observed with agonists that target either receptor class (CGS-21680, an AdR agonist and salmeterol, a β2AR agonist) when in combination with melphalan, lenalidomide, bortezomib or doxorubicin (Figure 1D and 1H, Figure S4A and
S4B). To determine whether the combination activity is cytostatic or cytotoxic, MM.1S cells were treated with individual agents or drug combinations and at various times and cells were analyzed by flow cytometry to determine the percentage of cells that express the apoptotic marker annexin V. As shown in Figure S5A, treatment of cells with 20nM CGS-21680 (AdR agonist), 0.13nM salmeterol (β2AR agonist) or 20nM Dex for 24 or 48 hours had minimal effects on cell viability. In contrast, substantially greater induction of apoptosis was observed when either of these agonists was used in combination with Dex, with most of the apoptosis occurring within 24 hours of drug exposure. The combinations containing CGS-21680 or salmeterol with lenalidomide (Figure S5B) or bortezomib (Figure S5C) also demonstrated greater induction of apoptosis by the combination as compared to the single agents. As a more stringent test of drug treatment on cell viability, we examined the extent to which salmeterol and bortezomib affect the ability of RPMI-8226 MM cells to form colonies when used as single agents or in combination. Cells were exposed to drugs for 5 hours, washed, plated at low density in Methocult and incubated for two weeks to allow for colony formation. Salmeterol and bortezomib each individually had some effect, with enhanced activity observed when the drugs were used together (Figure S5D), consistent with proliferation and apoptosis assay results.

**Validation of the A2A adenosine and β2AR subtypes as multiple myeloma drug targets**

We observe AdR agonist combination activity with CGS-21680 (an adenosine receptor A2A subtype agonist), Chloro-IB-MECA (A3 receptor agonist), ADAC (A1 receptor agonist), HE-NECA (an AdR pan-agonist) and other AdR agonists (Figure S2B and data not shown). All are active in our assays. Although some agonists are preferentially selective for a given adenosine receptor subtype, selectivity is lost when the compounds are tested at increasing concentrations due to the relatedness of the receptor subtypes and the cross-reactive nature of the compounds. We have used adenosine receptor subtype selective antagonists and siRNA, to demonstrate that the A2A receptor subtype (A2AR) is singularly responsible for AdR agonist antiproliferative effects (19).
We took the same approach to unequivocally demonstrate that β2AR is important for activity and synergy. Using MM.1S cells, 0.25μM lenalidomide was combined with 5nM clenbuterol (a potent β2AR agonist) and combination activity observed. Addition of the β2AR antagonist 0.9μM butoxamine did not affect lenalidomide activity but reduced clenbuterol single agent and combination activity (Figure S6A, B). Increasing the butoxamine concentration to 9μM abolished clenbuterol activity without reducing the lenalidomide effect. Similar results were obtained with a second selective β2AR antagonist, ICI 118,551 (Figure S6C). As additional validation, two different siRNAs targeting the β2AR receptor reduce the anti-proliferative activity of the β2AR agonist levalbuterol, but not an siRNA targeting A2AR (Figure S6D). Finally, neither the beta 1 agonist dobutamine nor the beta-3 agonist BRL 37344 have anti-proliferative activity as single agents or in combination with Dex at target-relevant concentrations, consistent with the β2AR receptor subtype as the target of interest (data not shown).

**Breadth of activity and selectivity**

To further examine A2AR and β2AR agonist selectivity and breadth, we evaluated combination activity in a panel of 83 cell lines including solid tumor types and hematological malignancies. Single agents and combinations with Dex and melphalan were systematically studied at multiple ratios of clinically relevant concentrations. Using a quantitative synergy score based on the Loewe model (13,15), we observe that combination activity for A2AR or β2AR agonists is highly selective for hematologic malignancies with synergy observed most frequently in multiple myeloma and DLBCL cell lines (Figure 2 and S7). With A2A agonists, synergy is also observed with the B-cell lines JM-1 (pre B-ALL) and GA-10 (Burkitt’s lymphoma). Using a relative synergy cut-off (synergy score >1), we find that 13 of the 18 MM cell lines tested demonstrate a synergistic interaction between the A2AR agonist CGS-21680 and Dex (Figure 2A) and 11 of 18 demonstrate a synergistic interaction between CGS-21680 and melphalan (Figure S7A). Using this same measure, 9 of 18 MM cell lines demonstrate synergy with combinations of the β2AR agonist salmeterol with either Dex or melphalan (Figure 2B and S7B). Eight and 6 of the cell lines in this MM panel were insensitive or responded weakly to Dex (<50% inhibition at 0.225μM) and
Adenosine A2A and Beta-2 Adrenergic Receptor Agonists: Novel Selective and Synergistic Multiple Myeloma Targets Discovered through Systematic Combination Screening

melphalan (<50% inhibition at 10uM) respectively when assayed as single agents. All cell lines were treated with the same concentrations of Dex or melphalan, pointing to A2AR agonists having a higher breadth of activity across the MM cell line panel than β2AR agonists. An intriguing observation is the strong synergy observed for A2AR or β2AR agonists with dexamethasone in the glucocorticoid-insensitive cell lines EJM and ANBL-6, which suggests that these agents may help restore steroid sensitivity in refractory patients (Figures 2 and S3). Combination effects were not observed when CGS-21680 or salmeterol were used in combination with Dex or Bortezomib in non-transformed cells (Figure S8).

**Analysis of A2AR and β2AR agonist activity in antitumor models ex vivo and in vivo**

We examined the combination activity of the A2AR agonist CGS-21680 and β2AR agonist salmeterol in combination with Dex using CD138+ MM cells isolated from the bone marrow of four patients. Tumor cells were incubated with clinically relevant concentrations of single agents or combinations for 48 hours and viability determined (Figure 3). Tumor cells from all four patients were less viable when treated with A2A or β2AR agonists and Dex vs. the single agents. Patients 1 and 3 had the best response. Cells from one patient were treated with A2AR or β2AR agonist and bortezomib. In general, activity was better for the drugs in combination than as single agents.

Single agent and combination activity was examined in xenograft models using three different MM cell lines (Figure 4 and Table 1). In MM.1S tumor xenografts, either the A2AR agonist CGS-21680 or the β2AR agonist salmeterol, when combined with either Dex or bortezomib, demonstrated significant reduction in tumor growth and enhanced survival that were greater than any single agent alone. In H929 xenografts, while little difference in tumor growth compared to single agents was observed at study day 20, the combination of CGS-21680 and Dex continued to inhibit tumor growth through the remainder of the study and conferred a significant survival advantage compared to either agent alone. Notably, RPMI-8226 xenografts were very sensitive to both dexamethasone and bortezomib, limiting the opportunity to enhance the effects of high doses of these drugs. However, salmeterol was able to enhance the effect of a sub-therapeutic
dose of bortezomib in this model. Similarly in MM.1S cells, addition of CGS-21680 to a sub-therapeutic dose of bortezomib results in reduction in tumor volume similar to higher doses of bortezomib alone. Interestingly, while Dex and bortezomib demonstrated expected levels of single agent activity in these models, CGS-21680 and salmeterol alone showed modest activity at best. Importantly, the addition of either CGS-21680 or bortezomib resulted in no additional toxicity in these models as measured by body weight change.

**Transcriptome analysis**

As a first step towards understanding the multi-target mechanisms important for combination drug synergy, we generated gene expression profiles for MM.1S cells either not treated, or after 6hr incubation with single agents or combinations. Supervised hierarchical analysis was performed to identify genes upregulated or downregulated by combination drug treatment (Figure S9). Some potential mechanism-relevant drug combination-specific gene expression changes are highlighted in Figure 5. The transcription factors MAF, IRF4 and MYC are all downregulated upon combination treatment. The rate-limiting metabolic enzymes HK2, PDK1, CYCS, SCD and FASN are also downregulated, as is CDK6, important for cell cycle progression. Upregulation of the pro-apoptotic gene BCL2L11 (BIM) and reduction of anti-apoptic BCL2L1 (BCL-XL) is observed. TSC22D3 (GILZ) a leucine zipper transcription factor is upregulated by combination drug treatment.

**Discussion**

Using cHTS, we have discovered that agonists of either the adenosine A2A or beta-2 adrenergic receptor have potent synergistic antiproliferative activity when used in combination with Dex (Figures 1, S2B,C and S3A,B). Induction of apoptosis is rapid and occurs within 24 hours of drug treatment (Figure S5). Synergistic combination activities are also observed when A2AR or β2AR agonists are crossed with the MM drugs melphalan, lenalidomide, bortezomib and doxorubicin at clinically relevant concentrations (Figures 1D and H, S4A,B). The adenosine A2A and beta-2 adrenergic receptors are essential for activity as antagonists or siRNA specific for the A2AR or β2AR block agonist effects (ref 19.
Adenosine A2A and Beta-2 Adrenergic Receptor Agonists: Novel Selective and Synergistic Multiple Myeloma Targets Discovered through Systematic Combination Screening

Greater than additive drug combination activities are observed with cell lines, patient tumor samples cultured ex vivo (Figure 3) and MM xenografts (Figure 4 and Table 1).

We screened 83 cell lines and observed that A2A and β2AR agonists combination activities are highly selective for hematologic malignancies with synergy observed most frequently in multiple myeloma and DLBCL cell lines (Figures 2 and S7). The analysis of additional cell lines and combinations may point to wider utility of these agents for cancer treatment. In our analysis, A2AR agonists had a highest breadth of activity across the 18 MM cell line panel. The MM cell lines differ with respect to sensitivity to agonists as single agents, which may help explain the lack of combination activity in some cell lines.

MM is currently an incurable disease. While many patients respond to one or more existing drugs, with time, the generation of resistance and ultimately relapse are likely. An interesting observation is the strong synergy observed for A2A or β2AR agonists with Dex in the GC-insensitive cell lines EJM and ANBL-6 (Figure 2 and S3A,B) which suggests that these agents may help restore steroid sensitivity in refractory patients. However, this is not true for the Dex-insensitive cell line MM.1R and the mechanism of drug insensitivity probably determines whether combination activity will be observed. Another interesting property of A2AR and β2AR agonists is their ability to increase the maximal efficacy and potency of MM drugs. Potency shifts can be particularly important for drugs that have significant adverse effects that limit their use. Bortezomib is one such drug, which is difficult to dose near the MTD for long durations due to peripheral neuropathy, and dose reductions are frequent in older more fragile patients (21). The use of bortezomib in combination with A2AR or β2AR agonists may allow a reduction in dose (and adverse effects) without loss of efficacy. This hypothesis is supported by the observation that in MM.1S xenografts, the addition of CGS-21680 to a sub-optimal dose of bortezomib (0.5 mg/ml) is able to confer a level of activity similar to that of a higher, more effective dose (0.8 mg/ml).

As our 18 MM cell line panel is unlikely to fully capture the genetic complexity found in patients, it was important to examine patient tumor cells A2AR and β2AR drug sensitivity and combination activities ex vivo. Our analysis of patient tumor
samples with CGS-21680 or salmeterol plus Dex (three patients) and CGS-21680 or salmeterol plus bortezomib (one patient) confirms that combination activity can also be observed ex vivo (Figure 3). In general, our results suggest that sensitivity of tumor cells to single agents increases the likelihood of combination activity. The combination effects observed, including absence of antagonism with SOC drugs suggests that A2A and β2AR agonists may provide benefit if added to current approved drug regimens.

As a first step towards understanding the molecular basis of combination drug synergy, microarray analysis was performed to study drug induced gene expression changes. The analysis provides insight into how the combination drugs potently kill MM cells. We find that IRF4 is downregulated, an essential gene that when silenced by RNA interference results in the rapid cell death of MM cell lines (22). MYC, a direct target of IRF4, is also downregulated. Like IRF4, silencing of MYC in MM cells is toxic (22). RNA levels for some downstream targets of these transcription factors are also reduced. These genes play key roles in cell metabolism and growth and include HK2, PDK1 and CYCS (glycolysis), SCD (lipid synthesis) and CDK6 (cell cycle regulation). Gene set enrichment analysis (GSEA), using a 36 member gene set of IRF4 target genes (Figure S10) further supports IRF4 pathway effects upon combination drug treatment.

In addition to IRF4 and MYC, the transcription factor MAF is downregulated. Exogenous expression of dominant negative MAF inhibits MM proliferation (23). Combination drug treatment upregulates the leucine zipper protein GILZ (TSC22D3), to a great extent than with Dex alone. Previously, it has been shown that GILZ is upregulated when MM.1S cells are treated with GCs and that reducing expression with siRNA decreases GC-induced cell death (24). These observations make GILZ an attractive candidate contributor to combination activity. Other genes where combination drug-specific changes in gene expression may adversely affect cell survival include PIM-2, TGFB1, PRKD2, CSNK1E, CDKN1C, GADD45A, ID2, BCL2L11 and BCL2L1 (25-32). Follow up analysis of the temporal changes in gene expression after combination drug treatment will help distinguish primary from secondary events.
To our knowledge, our laboratory is the first to report the surprising discovery that A2A agonists synergize with oncology drugs to induce apoptosis in preclinical models of MM and other B-cell malignancies. Other promising therapeutic areas for A2AR agonist development include inflammation, reperfusion injury, sepsis and diabetic peripheral neuropathy (33,34). At present, the only FDA approved A2AR agonists are regadenoson and adenosine, for use in combination with a cardiac imaging agent for patients who are unable to exercise during cardiac stress tests (35). Regadenoson has a short half life, which makes it ideal for cardiac imaging as effects can be rapidly termination once the procedure has been completed. For use in treatment of MM and other B-cell malignancies, molecules with longer transit circulation times coupled with dosing regimens that provide adequate exposure will be required. Our results suggest that A2AR agonists can synergize with MM drugs at concentrations below those where cardiovascular effects arise, making them an attractive new class of drugs for the treatment of MM.

Numerous β2AR agonist drugs have been developed for treatment of asthma and COPD as they cause smooth muscle relaxation, resulting in the dilation of bronchial passages (36). Most of the newer β2AR agonists have been formulated for delivery by inhalation and are not optimized for systemic exposure. Interestingly, β2AR agonists are often paired with corticosteroids (Advair Diskus, the combination of salmeterol and fluticasone and Symbicort, the combination of formoterol and budesonide). While each drug has distinct effects (β2AR agonist as a bronchodilator and corticosteroids anti-inflammatory), there may be beneficial synergy between these two classes of medication (37). Corticosteroids can enhance the expression of β2AR, which may provide some protection against desensitization and development of tolerance to agonists. Beta-2 adrenergic agonists may amplify the anti-inflammatory effects of corticosteroids by facilitating nuclear translocation of the GC receptor complex, and enhance transcription and expression of steroid-inducible genes in pro-inflammatory cells (38). The plethora of β2AR agonist drugs suggest a quick path to potential proof of concept MM clinical studies.
In recent years, drug discovery efforts have focused on the identification of agents that modulate validated therapeutic cancer targets (39-41). Given the complexity of roadblocks to cancer drug effectiveness, it is easy to see why the identification and deployment of combination therapies is such an important endeavor. The plasticity of tumor cell responses to drugs that affect growth and survival is best corralled using multi-targeted therapies. Cancer cells have less opportunity to adapt and drug synergies are most likely to reduce tumor load. An additional benefit is that synergistic combinations of two or more drugs may improve therapeutically relevant selectivity and reduce the toxicity and other side effects associated with high doses of single drugs (14). The exquisite selectivity of A2AR and β2AR synergies for tumor cells of the B-cell lineage support the idea that context dependent synergy can confer selectivity. Our use of a robust cHTS screening platform allows the analysis of many drug pairings to identify interesting combination effects and the determination of breadth of activity in cancers with different genetic backgrounds. The characterization of lead combinations in disease-relevant secondary assays such as ex vivo, microenvironment and immune models will facilitate the identification of novel cancer combination therapies with increased chance of selectivity and translation.

Acknowledgements

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Reference List

Adenosine A2A and Beta-2 Adrenergic Receptor Agonists: Novel Selective and Synergistic Multiple Myeloma Targets Discovered through Systematic Combination Screening


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Table 1. Summary xenograft studies. The activity of A2A AdR agonist CGS-21680 and β2AR agonist salmeterol in combination with multiple myeloma standard of care agents Dex and bortezomib. Activity was examined in three xenograft models.
Figure Legends

Figure 1. Adenosine receptor and β2AR agonists strongly synergize with dexamethasone and other standard of care drugs to inhibit multiple myeloma cell proliferation. A,E) A 9x9 dose matrix was generated for adenosine receptor agonist CGS-21680 x dexamethasone (A) and β2AR agonist salmeterol x dexamethasone (E). The matrix samples all mixtures of two serially diluted single agent concentrations. Inhibition is visualized using a color scale. The data in (A) is the average of six dose matrix while (E) is the average of two dose matrix. Control wells (no drug addition) are not part of the 9x9 dose matrix and are elsewhere on the 384 well plates. As a consequence, the “no drug” well in the matrix (lower left corner) inhibition values do not necessarily equal zero. B,F) The dose response observed for dexamethasone in the dose matrix is plotted (black line). Adding 6.5nM CGS-21680 (B, red line) or 0.4nM salmeterol (F, red line) to dexamethasone increases both potency and efficacy. C, G) Isobologram analysis of the dose matrix data for CGS-21680 x dexamethasone (C) and salmeterol x dexamethasone (G). Drug concentrations are plotted as fractions, so for CGS-21680, 1 = 0.019uM and dexamethasone, 1 = 0.021uM (panel C), salmeterol, 1 = 0.00021uM and dexamethasone, 1 = 0.018uM (panel G). D,H) Combination activities for AdR agonist CGS-21680 and β2AR agonist salmeterol with MM standard of care drugs. CGS-21680 and salmeterol were crossed with MM standard of care drugs bortezomib, dexamethasone, doxorubicin, lenalidomide and melphalan in MM.1S cells using the 9x9 dose matrix format (see Figures 3SA, B for representative data). The Combination Index (CI) was determined at the effect levels listed (CI%l). The CI is an estimate of how much drug was needed in combination relative to the single agent doses required to achieve the chosen effect level and CI values < 1 are synergistic. See Figure S1 legend for additional information.

Figure 2. 9x9 dose matrix screen of 83 tumor cell lines to determine selectivity and breadth of activity of A2AR and β2AR agonists in combination with
dexamethasone. A 9x9 dose matrix was generated as described in Figure 1. A) CGS-21680 and dexamethasone combination screening. Analysis of CGS-21680 started at 0.2uM with 2 fold dilutions, dexamethasone started at 2uM with 3 fold dilutions (see Figure 1A). B) The starting concentration for salmeterol was 5nM (2 fold dilutions) and 2uM for dexamethasone (3 fold dilutions). There were 2-4 replicates for each assay. To assess synergy, each test point is compared to the dose-additive model (expectation for a drug crossed with itself) that is calculated at every test point in the matrix using the single agent responses at the edges. See Supplemental Figure 1A for additional details. BCM = non-MM B cell malignancies.

Figure 3. A2AR and β2AR agonist combination activity in patient tumor cells cultured ex vivo. A) Sensitivity of primary MM tumor cells to CGS-21680, salmeterol and dexamethasone as single agents and in combination (patients 1-3). B) Sensitivity to CGS-21680, salmeterol and bortezomib as single agents and in combination (patient 4). The results for the agonists and Dex single agent and combination are the mean and standard deviation from 3-6 replicate measurements while with bortezomib/Dex, 2 replicate measurements.

Figure 4. In vivo activities of the A2A AdR agonist CGS-21680 and the β2AR agonists salmeterol in combination with Dex using an MM.1S xenograft model. Mice (6-7 per group, see Table 1) were challenged subcutaneously with MM.1S cells and treated with the various single agents and combinations indicated. See Materials and Methods for additional details.

Figure 5. Transcriptional profile for select genes upregulated or downregulated by combination drug treatment. The MM1.S cell line either untreated (lanes 1 and 2) or treated for six hours with 1nM Salmeterol (lanes 3 and 4), 12.5nM CGS 21680 (lanse 5 and 6), low dose dexamethasone (25nM, lanes 7 and 8), high dose dexamethasone (2μM, lanes 9 and 10), or combinations (CGS-21680 or Salmeterol in combination with low dose Dex, lanes 11-14). The data were normalized with Gene Pattern module ExpressionFileCreator using the GCRMA method. The color scheme in the heat map represents the relative expression level of the gene detected by the probe set in each row.
**FIGURE 1**

**A**  Inhibition

<table>
<thead>
<tr>
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<td>31.33</td>
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<tr>
<td>50</td>
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</tbody>
</table>

**B** Percent Inhibition

- **Vehicle**
- **CGS-21680 hydrochloride (0.156 µM)**

**C** Isobologram

- AdR Combination (CGS-21680)
- Bortezomib: 0.75, CI: 55
- Dexamethasone: 0.26, CI: 60
- Doxorubicin: 0.45, CI: 75
- Lenalidomide: 0.21, CI: 55
- Melphalan: 0.41, CI: 65

**E** Inhibition

<table>
<thead>
<tr>
<th>Dexamethasone (µM)</th>
<th>Salmeterol Xinafoate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0.04</td>
<td>0.07</td>
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</tbody>
</table>

**F** Percent Inhibition

- **Vehicle**
- **Salmetorol Xinafoate (2.1e-4 µM)**

**G** Isobologram

- B2AR Combination (Salmeterol)
- Bortezomib: 0.53, CI: 40
- Dexamethasone: 0.23, CI: 50
- Doxorubicin: 0.37, CI: 50
- Lenalidomide: 0.12, CI: 40
- Melphalan: 0.43, CI: 55
203085_s_at  "transforming growth factor, beta 1, TGFβ1"
213182_x_at  "cyclin-dependent kinase inhibitor 1C (p57, Kip2), CDKN1C"
216894_x_at  "cyclin-dependent kinase inhibitor 1C (p57, Kip2), CDKN1C"
219534_x_at  "cyclin-dependent kinase inhibitor 1C (p57, Kip2), CDKN1C"
201565_s_at  "inhibitor of DNA binding 2, dominant negative helix-loop-helix protein, ID2"
203725_at  "growth arrest and DNA-damage-inducible, alpha, GADD45A"
1553096_s_at  "BCL2-like 11 (apoptosis facilitator), BCL2L11"
1555372_at  "BCL2-like 11 (apoptosis facilitator), BCL2L11"
1558143_a_at  "BCL2-like 11 (apoptosis facilitator), BCL2L11"
222343_at  "BCL2-like 11 (apoptosis facilitator), BCL2L11"
225606_at  "BCL2-like 11 (apoptosis facilitator), BCL2L11"
207001_x_at  "TSC22 domain family, member 3, TSC22D3"
208783_s_at  "TSC22 domain family, member 3, TSC22D3"
204562_at  "interferon regulatory factor 4, IRF4"
202431_s_at  "v-myc myelocytomatosis viral oncogene homolog (avian), MYC"
206686_at  "pyruvate dehydrogenase kinase, isozyme 1, PDK1"
228452_at  "pyruvate dehydrogenase kinase, isozyme 1, PDK1"
202934_at  "hexokinase 2, HK2"
212218_s_at  "fatty acid synthase, FASN"
200381_s_at  "stearyl-CoA desaturase (delta-9-desaturase), SCD"
200382_s_at  "stearyl-CoA desaturase (delta-9-desaturase), SCD"
208905_at  "cytochrome c, somatic, CYCS"
208363_at  "v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian), MAF"
209347_s_at  "v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian), MAF"
209348_s_at  "v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian), MAF"
206665_s_at  "BCL2-like 1, BCL2L1"
212312_at  "BCL2-like 1, BCL2L1"
215037_s_at  "BCL2-like 1, BCL2L1"
204269_at  "pim-2 oncogene, PIM2"
202332_at  "casein kinase 1, epsilon, CSNK1E"
225756_at  "casein kinase 1, epsilon, CSNK1E"
226888_at  "casein kinase 1, epsilon, CSNK1E"
209282_at  "protein kinase D2, PRKD2"
38269_at  "protein kinase D2, PRKD2"
224848_at  "cyclin-dependent kinase 6, CDK6"
224851_at  "cyclin-dependent kinase 6, CDK6"
Molecular Cancer Therapeutics

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Mol Cancer Ther Published OnlineFirst April 3, 2012.

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