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Therapeutics, Targets, and Chemical Biology

Cancer Research

Preclinical Validation of a Single-Treatment Infusion Modality That Can Eradicate Extremity Melanomas

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Abstract

Isolated limb perfusion (ILP) with the chemotherapeutic agent melphalan is an effective treatment option for extremity in-transit melanoma but is toxic and technically challenging to deliver locoregionally. CBL0137 is an experimental clinical drug with broad anticancer activity in animal models, owing to its ability to bind DNA in a nongenotoxic manner and inactivate the FACT chromatin modulator essential for tumor cell viability. Here, we report that CBL0137 delivered by ILP in a murine melanoma model is as efficacious as melphalan, displaying antitumor activity at doses corresponding to only a fraction of the systemic MTD of CBL0137. The ability to bind DNA quickly combined with a favorable safety profile made it possible to substitute CBL0137 in the ILP protocol, using an intra-arterial infusion method, to safely achieve effective tumor suppression. Our findings of a preclinical proof of concept for CBL0137 and its administration via intra-arterial infusion as a superior treatment compared with melphalan ILP allows for locoregional treatment anywhere a catheter can be placed. *Cancer Res; 76(22); 6620–30. ©2016 AACR.*

Introduction

Melanoma is currently the fifth most common cancer in the United States, and its incidence continues to rise at a rate of approximately 2% per year (1). Following appropriate initial therapy, approximately 2% to 10% of extremity melanoma lesions recur as local in-transit metastases (2). In-transit metastases are located in the dermal and subdermal lymphatics distant from the primary lesion, place the entire extremity at risk, and are a potential precursor to distant metastasis (3, 4).

In-transit melanoma response rates to regional cancer therapies remain much higher than modern systemic therapies, including emerging immunotherapy agents (5). Compared with simple surgical resection of in-transit lesions, regional approaches treat an entire area at risk of recurrence from occult micrometastases. As it is "regional," cytotoxic agents can be delivered at doses several orders of magnitude higher than what can be safely achieved systemically (6, 7). One method for regional chemotherapy is isolated limb perfusion (ILP), in which the drug is circulated via the major artery and vein serving the affected extremity, while systemic blood flow is excluded using a tourniquet. ILP with the alkylating agent melphalan was first described as a treatment more than 50 years ago with the goal of targeting the affected limb, sparing extremity function, and limiting systemic toxicity (8). Less technically challenging methods of regional therapy have emerged, including infusion via percutaneously placed catheters (9). Melphalan remains the drug of choice for ILP, with complete response rates up to approximately 60% (10). Efficacy is increased by mild hyperthermia (infusion of drug solution at 42°C rather than 37°C) due, at least in part, to improved uptake of the drug by tumor tissue (11).

Despite the relative success of regional melphalan/hyperthermia in treating extremity in-transit melanoma, new therapeutic strategies with greater efficacy and safety are needed (e.g., partial leakage of melphalan into the systemic circulation can be highly toxic and a complete leak can be potentially lethal). Efforts have focused on agents that increase drug delivery to tumors, modulation of known resistance proteins, or targeting proteins in tumor cell survival or apoptotic pathways (12–14).

In the current study, we aimed to optimize and simplify the regional therapy of melanoma using a new anticancer agent, CBL0137, which has several distinct advantages over many other candidates, including rapid accumulation in tissues during first pass, simultaneous targeting of multiple cellular pathways that are critical for tumor cell survival and growth, and less toxicity toward normal cells. CBL0137 is the lead compound of a class of curaxins, carbazole-based small molecules that intercalate into DNA and change its topology without causing DNA damage. In CBL0137-treated tumor cells, the FACT chromatin remodeling complex (composed of SSRP1 and SPT16 subunits) becomes tightly associated with chromatin, and this leads to depletion of functional

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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FACT, activation of the p53 tumor suppressor (via phosphorylation by FACT-associated CK2) as well as inhibition of transcriptional programs dependent upon FACT (15, 16). Via FACT, CBL0137 regulates NF-κB and heat shock factor 1 (HSF1), which are critical to tumor cells (17). Together, the multiple pathways impacted by CBL0137-modulated FACT contribute to potent tumor suppression. Normal differentiated cells are largely impervious to the toxic effects of CBL0137 due to the absence of FACT expression and a reduced reliance upon FACT-dependent pathways compared with tumor cells. *In vivo* antitumor efficacy of CBL0137 has been demonstrated in multiple preclinical models, including colon, renal cell, non–small cell lung, head and neck squamous cell, breast, and pancreatic cancer (15, 18, 19). Herein, we utilized a highly aggressive murine B16 melanoma model to assess the potential of CBL0137 as an agent for regional melanoma therapy.

Materials and Methods

Chemicals and reagents

CBL0137 (lot 10-106-88-30, Aptuit) in 5% dextrose was generously provided by Incuron LLC. Melphalan and lipopolysaccharide (LPS) was purchased from Sigma-Aldrich and luciferin from Promega.

Tumor cells

B16 melanoma cells (tested and authenticated) were obtained from ATCC (2006). NF-κB–responsive luciferase reporter construct was utilized for B16 cells lentiviral transduction (B16 NF-κB-Luc cells; ref. 20).

In vitro cytotoxicity assays

B16 melanoma cells were plated at 5.0×10^4 cells (96-well plates). Twenty-four hours later, cells were treated with vehicle [5% dextrose:PBS (1:1)], CBL0137 (0.7, 1.4, 2.8, or 5.6 μmol/L), or melphalan (70 or 140 μmol/L) for 15 or 30 minutes; each condition tested in 10 replicates. Compounds were removed from wells, and cells weer washed with PBS. Viability was measured 3 hours after initiation of treatment by CytoSelect MTT Cell Proliferation Assay (Cell Biolabs, Inc.).

In vitro NF-κB luciferase reporter assay

B16 NF-κB-Luc reporter cells were treated with 5 or 10 μmol/L CBL0137 for 15 minutes, washed, and then incubated with 100 ng/mL *Escherichia coli* LPS for 6 hours. Luciferase activity was measured via Bright Glo Luciferase Assay (Promega) and a VICTOR X3 plate reader (PerkinElmer).

Mice

Female C57BL/6 mice (6–8 weeks old) were purchased from the NCI (Frederick, MD). All experimental protocols were approved by the Roswell Park Cancer Institutional Animal Care and Use Committee.

Tumor establishment

Mice were injected subcutaneously in the lateral aspect of the left distal hind limb with B16 melanoma cells (or in some experiments B16 NF-κB-Luc reporter cells). Cells (1×10^5) per injection were delivered in 0.1 mL of PBS. Treatments initiated when tumors reached 5 mm in their longest dimension (~7 days).

Treatment of B16 tumor–bearing mice

ILP. The mouse ILP procedure that recapitulates clinical parameters was employed (21). Targeted drug delivery occurs without leak into the systemic circulation (Supplementary Fig. S1). ILP flow rate was 0.15 mL/minute for 15 or 30 minutes at either 37°C or 42°C. CBL0137 perfusions over 15 minutes were performed with 0.044, 0.111, 0.222, and 0.444 mg/mL CBL0137 solutions, and perfusions over 30 minutes were performed with 0.022, 0.055, 0.111, and 0.222 mg/mL CBL0137 solutions. Melphalan (40 or 20 μg/mL) was perfused for 15 or 30 minutes, respectively.

Systemic administration. Systemic treatment groups received 80 mg/kg CBL0137 in 5% dextrose delivered via single or repetitive (once weekly for up to 4 weeks) slow (15–20 seconds) intravenous injection via tail vein.

Intra-arterial administration. CBL0137 (2, 4, 6, 8, and 12 mg/mL) was infused over 15 minutes at room temperature via the superficial femoral artery (without a tourniquet or collection of venous drainage as in ILP).

Assessment of toxicity

Established local toxicity grades (21) were assigned to each perfused limb. Reactions were evaluated daily, and the greatest toxicity observed was recorded. Morbidity was measured via a modified Wieberdink scale (22). Serum creatine kinase (CK) levels served as a marker of muscle toxicity. Blood (50 μL) was obtained from each mouse 24, 48, and 72 hours after treatment using the superficial temporal vein (23). CK levels were measured in serum samples using a VITROS 5.1 FS instrument (Ortho Clinical Diagnostics, Inc.).

Assessment of tumor responses

Three perpendicular axes of the tumors were measured every other day using external digital calipers (Control Co.) consistently by the same investigator. Tumor volume equaled $1/2 \times$ length $\times$ width $\times$ height. Tumor responses were assigned on day 28 after treatment by the following: progressive disease (PD) $\geq$ 25% increase in tumor volume compared with tumor volume at the start of perfusion; stable disease (SD) = tumor volume not substantially changed (within −25% to +25% range); partial response (PR) = decrease of tumor volume ranging from −25% to −90%; and complete response (CR) = decrease of tumor volume ranging from >90% (change of more than −90%) to undetectable. Mice that died or were euthanized due to morbidity, tumor ulceration, or tumor reaching the size endpoint (1,500 mm^3) prior to day 28 were classified as PD.

Detection of apoptotic cells

Nonsequential 9-μm thick sections were prepared (24) from tumors and normal muscle samples from the treated limb 72 hours after treatment. TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP–biotin nick end labeling) staining was performed by ApopTag Kit (Millipore Corporation). Fluorescence microscopy (Olympus) with a SPOT RT color camera (Diagnostic instruments, Inc.) quantified TUNEL-positive cells (at least 10 fields, unit area of each field = 0.34 mm^2) by measuring the area of TUNEL-positive cells using the ImageJ program (NIH, Bethesda, MD). Data presented as the percentage of total analyzed area occupied by TUNEL-positive cells.

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Western immunoblotting

Total soluble cellular protein extracts were prepared in RIPA buffer [150 mmol/L NaCl, 1% SDS, 10 mmol/L Tris (pH 8.0), 1% sodium deoxycholate, 1% NP-40] containing a protease inhibitor cocktail (Sigma-Aldrich). The following primary antibodies were used: goat anti-SSRP1 and rabbit anti-Srp16 (both from Santa Cruz Biotechnology), rabbit anti-p53 (Novocastra, Leica), and rabbit anti-Hsp70A1 inducible form (Assay Designs/StressGen). Rabbit anti-actin antibody was used to control for protein loading (Sigma-Aldrich). Detection of caspase-specific cleavage of PARP (antibody from Santa Cruz Biotechnology) analyzed apoptosis with ECL-Plus reagent for signal detection (PerkinElmer).

CBL0137 tissue concentrations

CBL0137 was extracted from homogenized tissue samples supplemented with 9 w/v parts of methanol, incubated overnight on a rocker at 4°C, and then centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was separated from precipitate and diluted (1:4) with 0.1% formic acid. Quantification of CBL0137 was achieved by LC/MS-MS using AB SCIEX QTRAP 5500 System (AB SCIEX).

NF-κB activity *in vivo*

CBL0137 was administered to tumor-bearing mice 30 minutes before LPS. A total of 2.5 hours after LPS injection, mice received intraperitoneal injection of 30 mg/mL luciferin solution and imaged for bioluminescence using IVIS Lumina III *in vivo* imaging system (PerkinElmer).

Statistical analyses

The therapeutic indices of cell viability, tumor volumes, CBL0137 tissue levels, serum CK levels, TUNEL data, and luciferase activity were assessed by Student *t* test. Tumor growth delay was assessed by ANOVA (GraphPad Prism, Version 6 software, GraphPad Software, Inc.). Values of $P < 0.05$ were considered significant.

Results

CBL0137 *in vitro*

Regional antimelanoma regimens in clinical use expose tumors to drugs in a single procedure (30–60 minutes) often combined with mild hyperthermia. This scenario was imitated *in vitro*, at 37°C or 42°C. Melphalan treatment for 15 minutes at clinically equivalent doses led to significant reduction of tumor cell viability at both 37°C and 42°C (Fig. 1A). Melphalan treatment for 30 minutes also reduced tumor cell viability at both temperatures; however, this was only statistically significant at 37°C, as at 42°C, vehicle-treated cells also demonstrated cell death. CBL0137 induced dose-dependent tumor cell death with 15, and to an even greater extent, 30 minutes of treatment. Decreased cell viability was statistically significant with respect to temperatures, times, and doses as shown in Fig. 1A.

As expected, based on prior observations (25), treatment of B16 cells with CBL0137 for 1 hour led to reduction of SSRP1 levels in soluble protein extracts (Fig. 1B). CBL0137 treatment also suppressed transcription of HSF1/hsp70 and stabilized p53 (Fig. 1C). CBL0137 treatment effect on NF-κB activity was evaluated using a variant of the B16 melanoma cell line carrying a NF-κB–regulated luciferase reporter transgene (B16 NF-κB-Luc cells; ref. 26). Cells were pretreated with either CBL0137 (5 or 10 μmol/L) or vehicle for 15 minutes. CBL0137 dose-dependent suppression of NF-κB pathway activity was observed (Fig. 1D).

Together, these results indicated that B16 melanoma cells are responsive to short-term exposure to CBL0137 and provided a foundation for testing CBL0137 *in vivo*.

Toxicity of CBL0137 ILP

To determine an effective dose for ILP, B16 tumor–bearing mice were treated for 30 minutes at either 37°C or 42°C with 0.1, 0.25, 0.5, or 1 mg (systemic MTD is ~2.0 mg) CBL0137 (total amount pumped through isolated mouse limb). For comparison, single intravenous MTD of CBL0137 in mice is approximately 120 mg/kg or approximately 3 mg total amount administered to a 25 g mouse. Melphalan (90 μg; single intravenous MTD is ~3.6 mg/kg for this drug) was used for comparison. Scoring of local toxicity on days 1 to 9 posttreatment revealed that toxicity was limited to grade 1 or 2 in all ILP-treated groups except for those that received the highest dose of CBL0137 (1 mg; Fig. 2A). No morbidity was noted in the vehicle or low-dose CBL0137 groups (0.1 and 0.25 mg); however, leg limping and skin necrosis were detected in high-dose groups (0.5 and 1 mg). At the 1 mg dose, grade 3 or 4 toxicity was observed in 85.7% of mice treated at 37°C and in 100% of mice treated at 42°C. Grade 2 toxicity became apparent shortly after ILP, but resolved in the majority of mice within 2 days. Temperature increase (42°C) did not affect regional toxicity of CBL0137 at 0.1, 0.25, and 0.5 mg, but increased the severity of toxicity associated with the 1 mg dose. Thus, we defined single ILP MTD of CBL0137 as 0.5 mg.

Elevation of serum CK levels, reflective of muscle damage (the most common clinical toxicity), was generally similar in mice subjected to ILP with MTD of either melphalan or CBL0137 (Fig. 2B). With both drugs, CK levels peaked at 48 hours after 15-minute ILP under normothermic (37°C) or hyperthermic (42°C) conditions and showed a trend toward resolution at 72 hours. The highest CK levels were detected 24 hours after 30-minute ILP under hyperthermic conditions. The comparison of CK levels after 15- and 30-minute ILP revealed direct proportionality between muscle damage extent and perfusion time. Importantly, although the ILP treatments caused elevation of serum CK beyond the "normal" range (<200 U/L), peak CK levels remained substantially below what is considered the "severe toxicity" range (>2,000 U/L) in all groups treated.

These studies demonstrated that doses of CBL0137 less than 0.5 mg given via ILP did not cause substantial toxicity in tumor-bearing mice and were suitable for efficacy testing.

Efficacy of CBL0137 ILP

Next, CBL0137 efficacy against limb B16 subcutaneous tumors was compared by route of administration. ILP was performed at either 37°C or 42°C to assess the possibility of enhanced anticancer effect due to CBL0137 inhibition of HSF1-dependent transcription and increased drug uptake with mild hyperthermia. Tumors grew rapidly in control mice that were untreated or given systemic vehicle (5% dextrose), reaching the endpoint size of approximately 1,500 mm^3 by day 10 after treatment (Fig. 3A). Single or once per week intravenous administration of CBL0137 at its once per week

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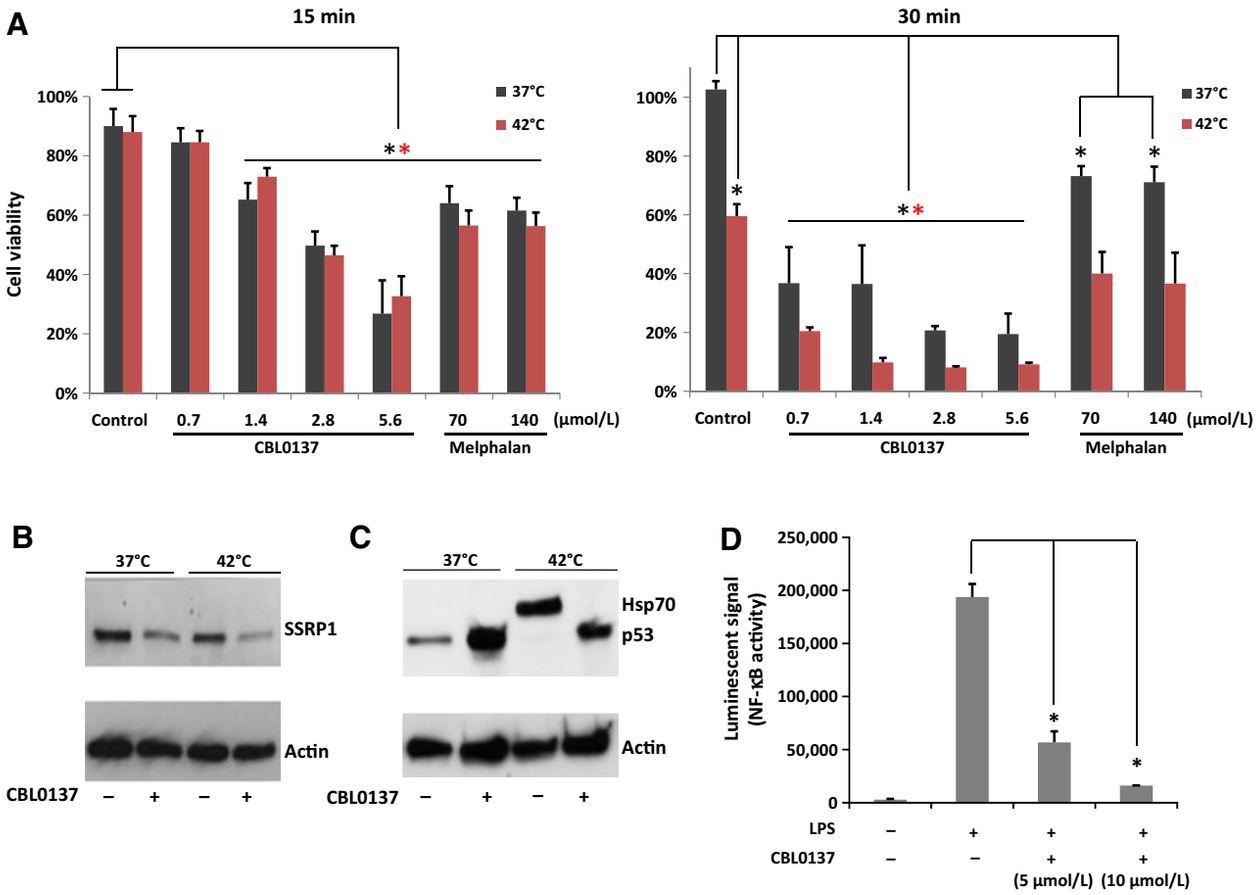

Figure 1.

CBL0137 *in vitro*. **A,** representative graph of B16 melanoma cells treated with CBL0137 or melphalan at 37°C or 42°C for 15 (left) or 30 minutes (right; triplicate experiments, mean ± SEM, 10 replicate wells; black asterisk, 37°C; red asterisk, 42°C; $P < 0.05$ by Student t test). **B** and **C,** representative Western blot analyses (duplicate experiments) extracts from B16 cells treated with 5 μmol/L CBL0137 or control for 1 hour at 37°C or 42°C. **B,** SSRP1 protein levels reduced by CBL0137 1 hour posttreatment. **C,** Hsp70 and p53 protein levels 6 hours posttreatment. **D,** luciferase activity of B16 NF-κB-Luc cell cultures treated with vehicle, LPS (100 ng/mL), and CBL0137 (5 or 10 μmol/L) as indicated (single experiment).

intravenous MTD (80 mg/kg) via the tail vein had minimal effect on the kinetics of tumor growth. In contrast, ILP delivery of 0.1 or 0.25 mg CBL0137 to the tumor-bearing extremity significantly suppressed tumor growth within 8 days compared with all other types of treatment tested. The antitumor effect of CBL0137 ILP appeared to be dose dependent, although the differences between doses were not statistically significant. At each dose, CBL0137 ILP under normothermic versus hyperthermic conditions resulted in similar tumor growth suppression. It is notable that a single 30-minute CBL0137 ILP treatment showed superior antitumor efficacy compared with systemic CBL0137 treatment, even when the total amount of perfused drug was only a fraction (12.5%) of the total amount of the drug administered systemically.

CBL0137 ILP regimens were then directly compared against melphalan, which is the most commonly used agent for clinical ILP. Single 30-minute ILP with either 0.1 or 0.25 mg CBL0137 or 90 μg melphalan resulted in comparable statistically significant tumor growth inhibition (Fig. 3B). A minor trend toward improved tumor growth control was observed when the lower CBL0137 dose was delivered under hyperthermic conditions. There was no significant difference in tumor growth between study groups treated with vehicle at 37°C and 42°C and corresponding untreated control groups.

We next determined whether shorter infusion times (15 vs. 30 minutes) could be performed with higher doses of CBL0137 to simplify clinical application. Fifteen-minute ILP had statistically significant antitumor effects, similar to those seen with 30-minute ILP, and again, trends toward dose- and temperature dependence of antitumor effect were observed (Fig. 3C).

Mice were categorized in terms of clinical response on day 28 after treatment. The proportion of mice in each treatment group showing CR, PR, SD, or PD are shown (Fig. 3D). Regional therapy with CBL0137 produced durable and complete responses in a dose-dependent manner that was enhanced by hyperthermic conditions (greater number of mice with CR with ILP performed at 42°C). In treatment groups exhibiting a CR, 33% to 66% of CBL0137-treated mice achieved a CR that was significantly greater than the approximately 13% obtained with conventional melphalan ILP. Furthermore, shorter duration perfusions with higher CBL0137 doses were equivalent to the longer duration with lower CBL0137 doses in terms of therapeutic responses achieved,

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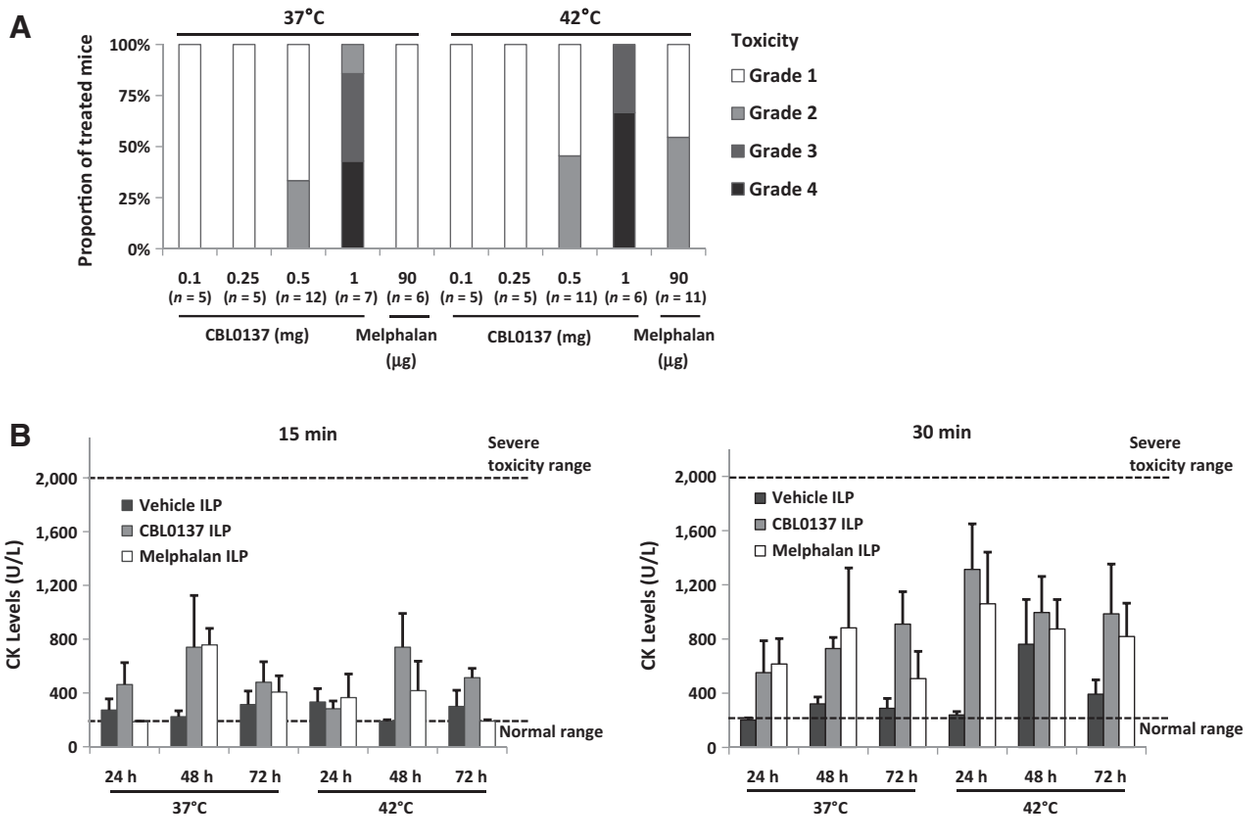

Figure 2.

Toxicity of CBL0137 ILP. **A,** local toxicity after ILP (30 minutes) at either 37°C or 42°C with 0.1, 0.25, 0.5, or 1 mg CBL0137 or 90 μg melphalan ($n = 5$–7 mice/group, pooled data). **B,** serum CK levels measured 24, 48, and 72 hours after 15- and 30-minute ILP at 37°C or 42°C with vehicle (control), 0.5 mg CBL0137, or 90 μg melphalan (mean ± SEM; $n = 6$ mice/group, pooled data). Dotted lines, range of toxicity (*, $P < 0.05$ by Student t test).

suggesting a technical advantage over standard clinical ILP durations of 30 to 60 minutes.

FACT activity of CBL0137 ILP

To confirm that the mechanism of action underlying the antitumor effects of CBL0137 in ILP with B16 melanoma is the same as that previously established *in vitro* (15) and in other *in vivo* tumor models (19), we probed the response of CBL0137 targets, FACT, p53 and NF-κB.

Soluble levels of both FACT subunits were reduced in tumors from CBL0137-treated mice compared with vehicle controls, and the reduction was greater with hyperthermic ILP (Fig. 4A). Examination of tumor extracts collected at a later time point (24 hours) after single 15-minute ILP with 0.5 mg CBL0137 showed persistent depletion of FACT subunits and prominent p53 stabilization in both CBL0137-treated groups (Fig. 4B). The effects of CBL0137 ILP (15 or 30 minutes) on FACT subunit levels and p53 activation were similar between groups subjected to normothermic or hyperthermic ILP.

To evaluate suppression of NF-κB–dependent transcription *in vivo*, mice were inoculated with B16 NF-κB-Luc cells. NF-κB–dependent luciferase expression was induced in tumor-bearing mice with LPS. The effect of CBL0137 treatment route on LPS-induced NF-κB activity in tumors was determined using whole-animal bioluminescent imaging (Fig. 5A). The strong luciferase expression induced by LPS was only mildly reduced by single 80 mg/kg intravenous injection of CBL0137 (total administered drug amount equivalent to 2 mg via ILP) but was completely or near-completely eliminated in mice treated with 0.5 mg CBL0137 delivered via ILP. No significant difference in inhibition of NF-κB activity following CBL0137 ILP was noted at either normothermic or hyperthermic conditions (Fig. 5B). Thus, CBL0137 ILP had a potent inhibitory effect on NF-κB activity in tumors compared with intravenous treatment even when the total amount of CBL0137 delivered via ILP was 4-fold lower than the one delivered intravenously.

As CBL0137 ILP stabilizes the proapoptotic p53 tumor suppressor, and inhibits antiapoptotic NF-κB signaling, its effect on apoptosis of tumor and normal cells was evaluated. TUNEL staining revealed variable tumor cell apoptosis in all groups (Fig. 6A), with 3% to 10% of apoptotic tumor cells seen with either CBL0137 or melphalan ILP at normothermic conditions. Hyperthermia significantly increased apoptotic tumor cells (~30%–40% of tumor cells with either CBL0137 or melphalan treatment). Importantly, combination of hyperthermia with ILP did not cause a significant increase in the degree of normal cell (muscle) apoptosis in the ILP-treated limb with either CBL0137 or melphalan (below 1%). Also, vehicle with hyperthermia did not affect the baseline level of apoptosis in either tumors or muscle.

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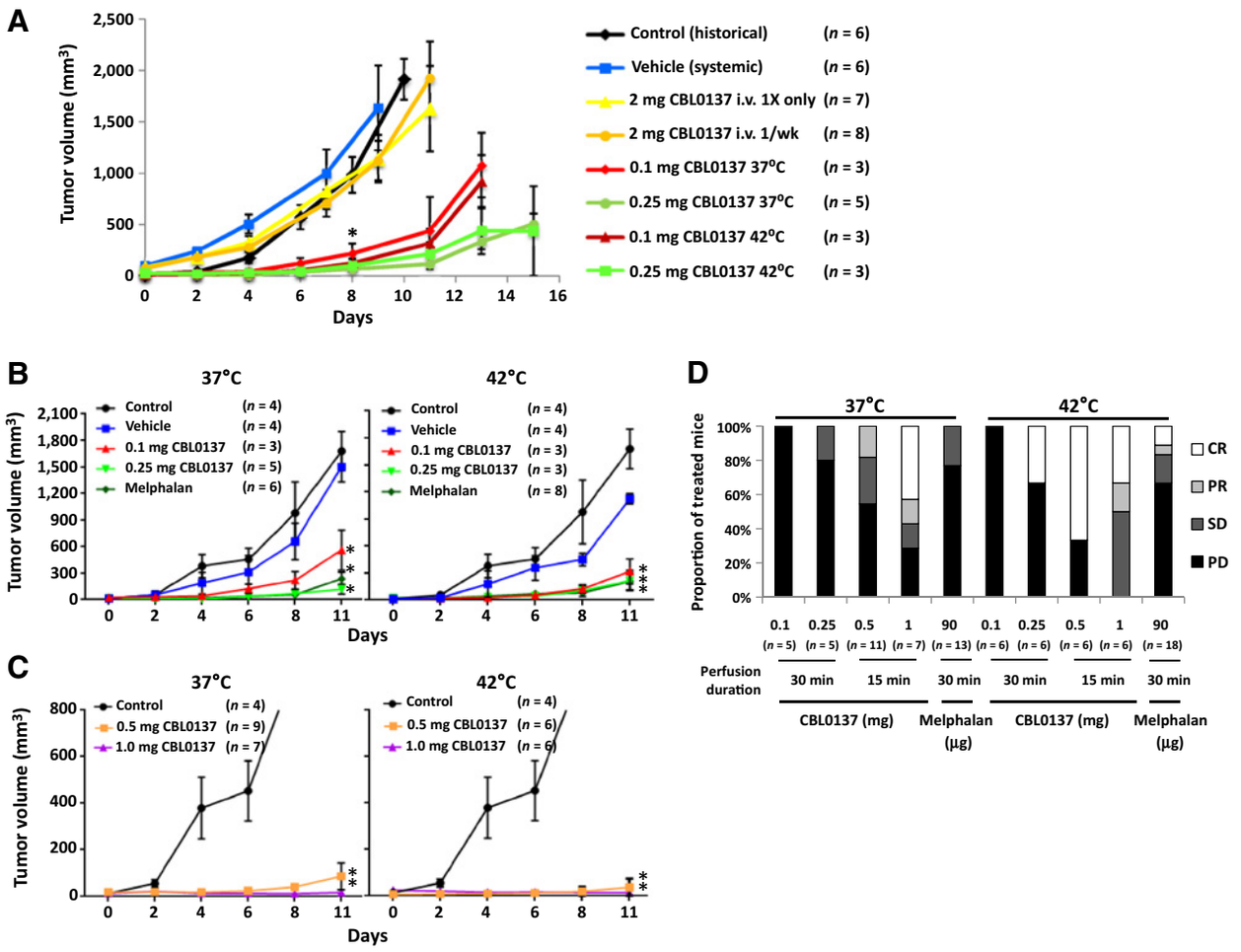

Figure 3.

CBL0137 *in vivo.* **A–C,** B16 melanoma tumor growth treated as indicated on day 0 (mean ± SEMs, pooled data; *, $P < 0.05$ by ANOVA). **A,** untreated mice (control), treated systemically [intravenous (i.v.) injection] with vehicle (5% dextrose) once or 80 mg/kg CBL0137 once or once per week, or treated by ILP (30 minutes) with 0.1 or 0.25 mg CBL0137 at 37°C or 42°C. **B,** untreated mice (control) or treated by ILP (30 minutes) with vehicle (5% dextrose: PBS, 1:1, v/v), 0.1 or 0.25 mg CBL0137, or 90 µg melphalan at 37°C (left) or 42°C (right). **C,** untreated mice (control) or treated by ILP (15 minutes) with 0.5 or 1 mg CBL0137 at 37°C (left) or 42°C (right). **D,** clinical responses on day 28 after ILP treatment (n = 5–18 mice/group, pooled data).

Caspase-mediated cleavage of PARP by Western blotting using protein extracts prepared from tumors collected 48 hours after ILP was confirmatory. Increased levels of cleaved PARP (PARPcl) were seen in tumors collected from CBL0137 groups at normothermic conditions compared with tumors from vehicle or melphalan treatment groups. With treatment at hyperthermic conditions, both CBL0137 and melphalan induced PARP cleavage. Stronger PARPcl signals were detected at hyperthermic compared with normothermic treatment conditions (Fig. 6B).

Therefore, CBL0137 effects on key factors responsible for its mechanism of activity appeared much stronger *in vivo* when delivered via ILP as compared with intravenously, indicating potentially greater clinical efficacy of regional CBL0137 delivery.

Tumor uptake of CBL0137

In the experiments described above, hyperthermia mildly improved the inhibitory effect of CBL0137 ILP on B16 melanoma growth in the perfused extremity. CBL0137 showed inhibitory effects on HSF1 in B16 melanoma cells *in vitro* and demonstrated improved efficacy with hyperthermia *in vivo*; therefore, we examined whether hyperthermia also increased CBL0137 distribution into tissues, as has been described for melphalan and other compounds delivered via ILP (11). B16-bearing mice were perfused for 30 minutes with 0.1 or 0.25 mg CBL0137 at normothermic or hyperthermic conditions, during which time, consecutive 500 µL aliquots of venous drainage were collected. CBL0137 concentrations were measured by LC/MS-MS in each aliquot of drainage fluid to monitor drug retention by tissues during ILP. At both CBL0137 doses, there was less drug in the drainage fluid when ILP was performed at hyperthermia compared with normothermia, indicating enhanced delivery/uptake of the drug into tissues at the higher temperature (Fig. 6C). The substantial drop in CBL0137 concentration between the dosing solution and the first aliquot of drainage fluid suggested rapid uptake of the drug into the

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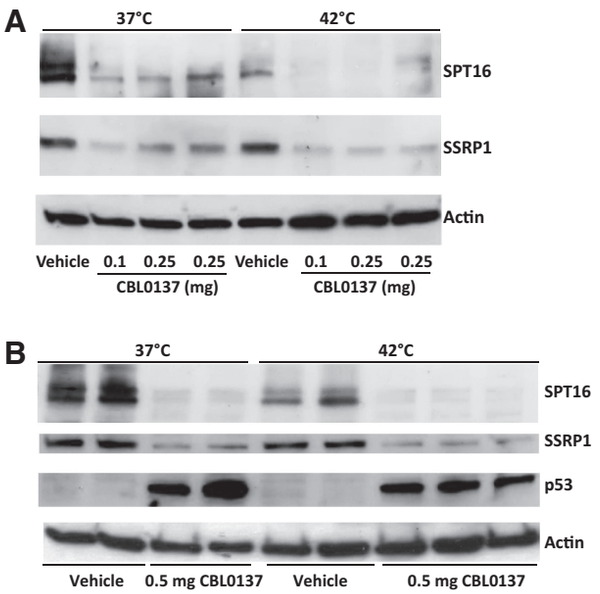

Figure 4.

FACT activity of CBL0137 ILP. **A,** representative Western blot analysis, antibodies against FACT subunits (SPT16 and SSRP1) and actin (loading control) with tumor protein extracted 1 hour after 30-minute ILP treatment with vehicle or CBL0137 (0.1 or 0.25 mg) at 37°C or 42°C (lane, individual tumors). **B,** representative Western blot analysis, including antibody against p53 with tumor protein extracted 24 hours after 15-minute ILP with CBL0137 (0.5 mg; replicate lanes of individual tumors, duplicate experiments).

tissue of the treated limb or, in other words, an efficient and profound "first-pass" effect. CBL0137 concentration went up in subsequent fractions of drainage fluid, indicating an established equilibrium between delivery and clearance of CBL0137.

CBL0137 ILP under hyperthermic conditions led to increased CBL0137 concentration in both tumor and muscle tissues from perfused limbs (Fig. 6D). Most importantly, ILP delivery promoted uptake of the drug specifically into tumor tissues: Levels of CBL0137 were approximately 100- and 1,000-fold higher in tumor tissue than in muscle tissue at 24 hours after ILP performed at normothermic and hyperthermic conditions, respectively (Fig. 6D). In contrast, intravenous treatment with CBL0137 resulted in a much lower ratio between CBL0137 concentrations in tumor tissue as compared with muscle. The ratio of tumor to muscle CBL0137 concentrations achieved after ILP and intravenous drug delivery suggested that the CBL0137 therapeutic index can be improved by selecting the ILP method of administration. This principle is supported by the previously mentioned tumor growth experiments, where systemic administration of CBL0137 was markedly inferior to regional administration in terms of tumor growth inhibition.

Intra-arterial infusion of CBL0137

On the basis of the findings that (i) there is a profound "first-pass" effect associated with ILP delivery of CBL0137 and (ii) antitumor efficacy was observed at a fraction (12.5%) of systemic MTD, we examined whether CBL0137 could be effective if delivered via intra-arterial infusion (IAI), without the application of a tourniquet or collection of venous effluent. The IAI methodology was investigated as it would be a much simplified technique to perform clinically, and it would represent a major advancement in regional therapy, as current cytotoxic agents cannot be given by simple infusion due to their excessive systemic toxicity.

To establish the MTD of CBL0137 for this IAI administrative route, mice were treated with a single 15-minute IAI of vehicle or CBL0137 (total amount of 0.5, 1, 1.5, 2, or 3 mg per infusion) and scored for clinical toxicity. Only the 0.5 mg CBL0137 group showed toxicity similar to vehicle (Fig. 7A). In general, serum CK levels rose following IAI of CBL0137, demonstrating an early peak at 24 hours posttreatment and resolution beginning at 48 hours (Fig. 7B). Toxicity of IAI CBL0137 treatment was dose dependent. Thus, the 3 mg dose caused excessive muscle toxicity, which 24 hours post-IAI was significantly higher compared with the toxicity of vehicle and low-dose groups. Maximal serum CK levels in the 0.5, 1, and 1.5 mg dose groups did not reach the level typically indicative of severe toxicity. Therefore, 0.5, 1, and 1.5 mg doses of CBL0137 were selected for further use in IAI antitumor efficacy experiments.

Mice were given a single 15-minute IAI of CBL0137 (0.5, 1, or 1.5 mg), resulting in significant tumor growth inhibition (Fig. 7C) comparable with prior results achieved with CBL0137 ILP. On day 12 posttreatment, mean tumor volumes in the 0.5, 1, or 1.5 mg CBL0137 treatment groups were 302.2, 200.4, and 236.2 mm^3, respectively, compared with 1,144.5 mm^3 in the vehicle-treated control group ($P < 0.04$ for all comparisons with vehicle group). Categorization of clinical responses 28 days posttreatment showed that whereas all vehicle-treated animals had PD, approximately one third of CBL0137-treated mice achieved CR, PR, or SD (Fig. 7D).

As the least toxic 0.5 mg dose of CBL0137 was as efficacious as higher doses with 15-minute IAI, we tested whether its efficacy was maintained at reduced IAI duration. Indeed, treatment of B16-bearing mice with 0.5 mg CBL0137 using 5-minute IAI resulted in tumor growth inhibition equivalent to that seen after 15-minute IAI of the same dose (Fig. 7E). This suggests that CBL0137 is rapidly absorbed into body tissues, including tumor tissue, and that IAI duration can be shortened without compromising efficacy. Concentrations of CBL0137 in tumor, plasma, muscle, and liver tissue samples collected from IAI or intravenously treated mice at room temperature were analyzed 6 and 24 hours after dosing. Significantly higher concentrations of CBL0137 were found in tumor tissues collected from IAI versus intravenously treated mice (Fig. 7F). CBL0137 concentrations in tumor tissue after IAI with 1 mg of CBL0137 were higher than after intravenous treatment with 80 mg/kg of CBL0137. This trend was persistent 24 hours after IAI treatment. In addition, lower CBL0137 concentrations were detected in normal tissue of the mice treated via IAI compared with intravenously treated mice.

Thus, as opposed to the more complex ILP technique, a single CBL0137 IAI demonstrated equal antitumor efficacy, toxicity, and tumor drug uptake but was much easier to perform.

Discussion

Regional therapy of in-transit melanoma metastases with the clinical standard melphalan has been employed for more

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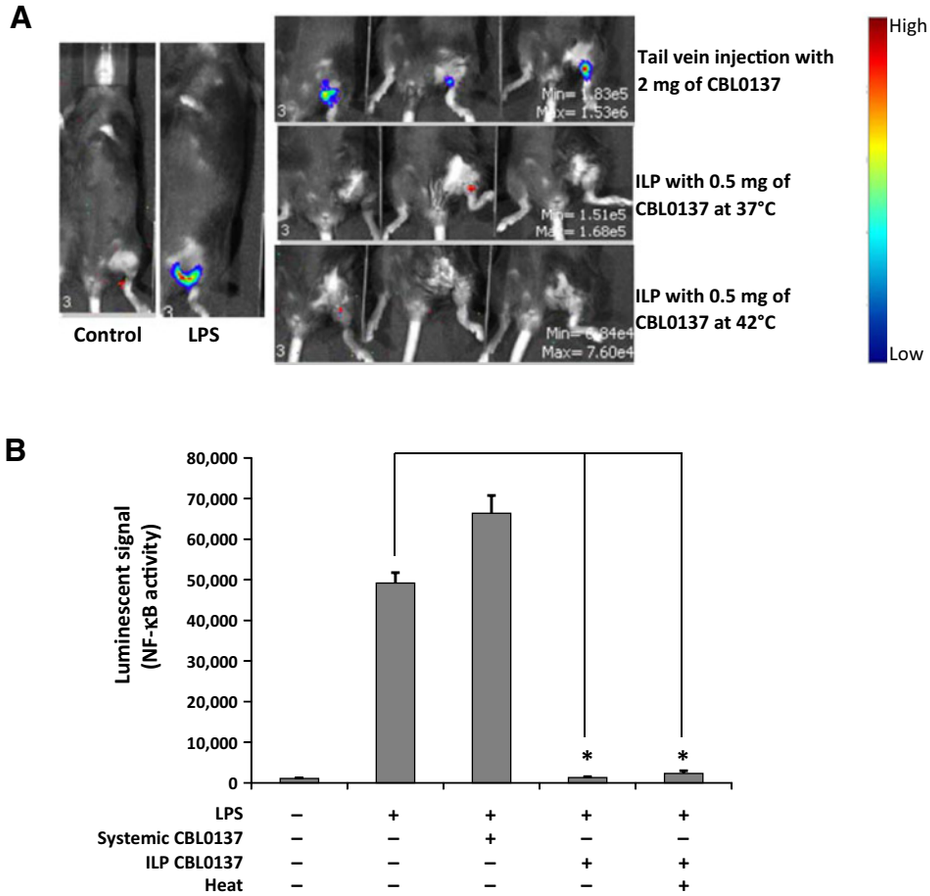

Figure 5.
NF-κB activity with CBL0137. **A,** representative photomicrograph of B16 NF-κB-Luc subcutaneous tumors of left lower extremity; untreated (control) or treated intraperitoneally with LPS (10 μg/mouse) alone or in combination with systemic CBL0137 (intravenously via tail vein, 2 mg) or ILP CBL0137 (15 minutes, 0.5 mg, 37°C or 42°C; $n = 3$ mice/group). **B,** quantification of *in vitro* luminescent signals with indicated treatments (mean ± SEM, $n = 3$ mice/group, single experiment; *, $P < 0.05$ by Student *t* test).

than 50 years, and to date, no other agent has proven to be clinically superior. Herein, we report therapeutic efficacy of a novel, nongenotoxic anticancer agent, CBL0137, equivalent to that of melphalan in a murine model of extremity melanoma. Unlike melphalan ILP, which requires high dosing, continuous monitoring for tourniquet leak, and the potential for significant systemic toxicities, CBL0137 was well tolerated and effective when given by a simple IAI. It is difficult to directly compare the doses of CBL0137 delivered via ILP and intravenously due to the restricted area of drug exposure in ILP as well as factors such as perfusion volume, flow rate, etc. However, even the lowest total amounts (0.1 mg) of CBL0137 delivered by ILP demonstrated superior antitumor efficacy compared with intravenous delivery of the drug at its MTD. CBL0137 is the first drug to challenge the standard paradigm for regional anticancer therapy that typically employs a cytotoxic agent, like melphalan, at much higher doses than can be delivered systemically requiring close monitoring of ILP circuits for leakage (27). As the dose of CBL0137 given by ILP was a fraction of the intravenous MTD, isolation of the treated limb is not necessary. With so little CBL0137 in the ILP circuit, even a complete leak should have no significant adverse consequences.

The antitumor effects of CBL0137 delivered by ILP were enhanced with the application of hyperthermia. Because of a unique mechanism of action, the basis for this effect may be fundamentally different for CBL0137 compared with melphalan. While ILP with melphalan under hyperthermic conditions increases vasodilation and drug uptake by tumors (28–32), hyperthermia similarly affected CBL0137 uptake by tumor tissue. However, in addition to this "nonspecific" effect, combination of hyperthermia and CBL0137 is likely to specifically enhance tumor cell killing due to CBL0137's ability to inhibit protective HSF-1–driven heat shock responses in tumor cells (19, 25, 33, 34). Hyperthermic ILP is expected to further induce heat shock responses in tumor cells. CBL0137 is anticipated to block this response, allowing tumor cells to undergo heat stress–induced apoptosis and improve efficacy.

ILP-delivered CBL0137 preferentially accumulated in tumor tissues compared with exposed normal tissues. CBL0137 tumor uptake occurred rapidly, and this significant "first-pass" effect was further enhanced by hyperthermia. This first-pass effect, together with the observed antitumor efficacy of small amounts of ILP-delivered CBL0137, suggested that CBL0137 might be safely and efficiently delivered by IAI. Although in theory, IAI is a systemic delivery method, we hypothesized that rapid uptake of the drug by the tumor tissue in the vicinity of the injection site would essentially make it a regional therapy. Indeed, IAI of CBL0137 at nontoxic doses without hyperthermia was clinically effective. IAI of CBL0137 could be effective against tumors in other locations as well, including any viscera or site of metastasis that can be accessed by an arterial catheter, thus potentially extending "regional" CBL0137 therapy to a broad range of metastatic clinical situations with greater safety and feasibility. Therefore, complex clinical techniques, including hepatic artery infusions, isolated pelvic perfusions, renal artery perfusions, and directed lung

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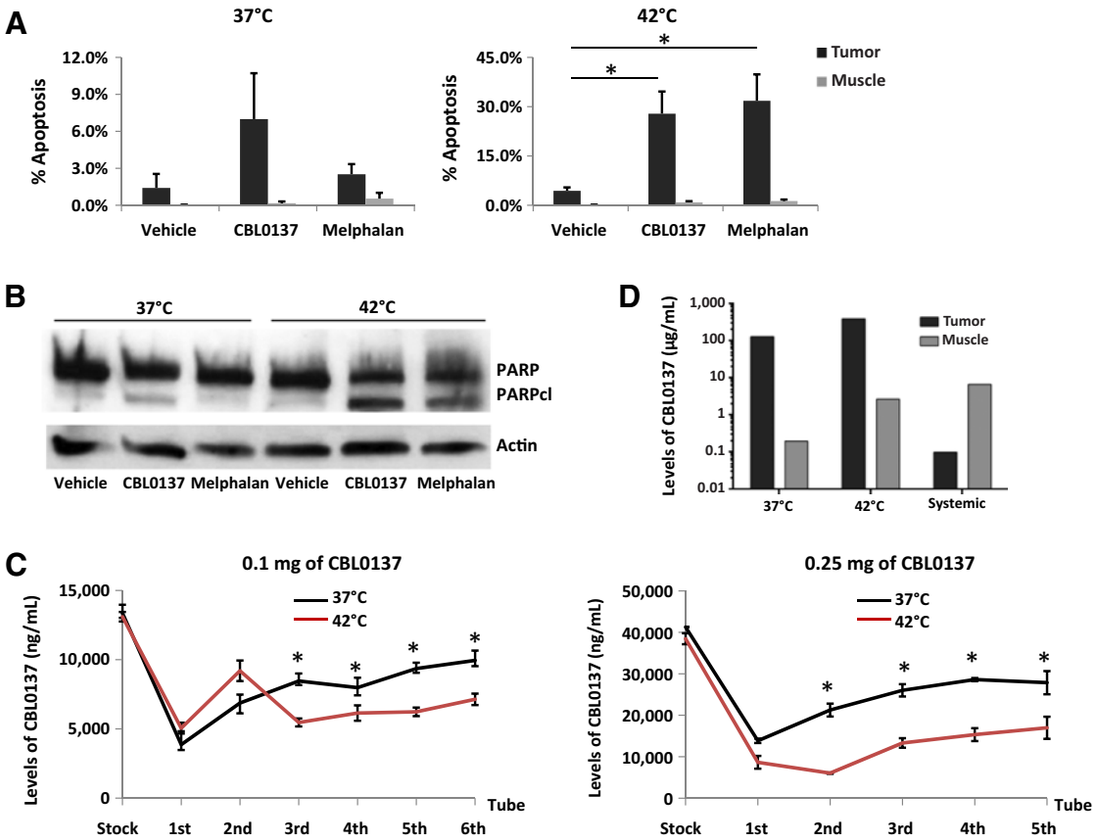

Figure 6.

CBL0137 tissue accumulation. **A,** percent apoptosis 72 hours after ILP (15 minutes) with vehicle, CBL0137 (0.5 mg), or melphalan (90 μg) at 37°C or 42°C (mean ± SEM; $n = 3$ mice/group; *, $P < 0.05$ by Student t test, single experiment). **B,** representative Western blot analysis of tumor extracts, antibodies against PARP and actin (loading control) 48 hours after ILP (15 minutes) with vehicle, CBL0137 (0.5 mg), or melphalan (90 μg) at 37°C or 42°C (lane, individual tumors, duplicate experiments). **C,** initial stock and collected aliquots of venous effluent during 30-minute ILP of B16 tumor–bearing mice; CBL0137 concentration determined by LC/MS-MS. ILP with 0.1 (left) or 0.25 mg CBL0137 (right; $n = 5$ mice/group; mean ± SEM; *, $P < 0.05$ by Student t test, single experiment). **D,** log scale of CBL0137 determined by LC/MS-MS collected 24 hours after CBL0137 ILP at 37°C, 42°C, or after systemic (intravenous) CBL0137 injection ($n = 1$ mouse/group, single experiment).

perfusion/suffusion, may be greatly simplified by using IAI of CBL0137 not only for potential equivalent antitumor efficacy, but also repeated applications.

Despite these promising results, additional work is needed to confirm the potential clinical usefulness of CBL0137 delivered via ILP or IAI. First, although our work demonstrated a profound effect on the highly aggressive murine melanoma cell line B16, it is unclear how CBL0137 would fare against spontaneously arising or long-established human tumors or how ILP and IAI would compare in head-to-head experiments in terms of efficacy and mechanisms. Second, due to the limitations of the mouse model, we only examined treatment regimens involving single ILP or IAI application of CBL0137. It is possible that in human patients, repeated treatments might generate better antitumor responses or, alternatively, greater tissue toxicities. In addition, the contributions of the immune system to CBL0137 efficacy remain unknown, but could be dissected in existing preclinical models and ultimately indicate synergy with emerging immunomodulatory agents.

In summary, this work provides a strong rationale for further investigation of ILP and IAI administration of CBL0137 for the treatment of metastatic cancer. CBL0137 was as efficacious as the ILP clinical standard melphalan with a similar toxicity profile in the murine model. However, advantages of CBL0137 over melphalan include the potential for greater synergy with hyperthermia due to CBL0137-mediated inhibition of heat shock responses, efficient first-pass uptake of CBL0137 into tumor tissue, and clinical efficacy at levels of CBL0137 that if escaped systemically would be a fraction of the systemic MTD. These features allowed CBL0137 to be administered by direct IAI instead of ILP. Such systemic administration is not possible with melphalan. Finally, the advanced developmental stage of CBL0137, currently in clinical trials as a systemic anticancer treatment, will benefit by exploration of the drug as a regional therapy for in-transit melanoma and, ultimately, other types of metastatic disease.

Disclosure of Potential Conflicts of Interest

K.V. Gurova reports receiving a commercial research grant from Incuron, has received speakers bureau honoraria from Incuron and Mayo Clinic, and is a consultant/advisory board member for Incuron. A.V. Gudkov is the chief scientific officer at and reports receiving a commercial research grant from Cleveland BioLabs, Inc., and has ownership interest (including patents) in and is

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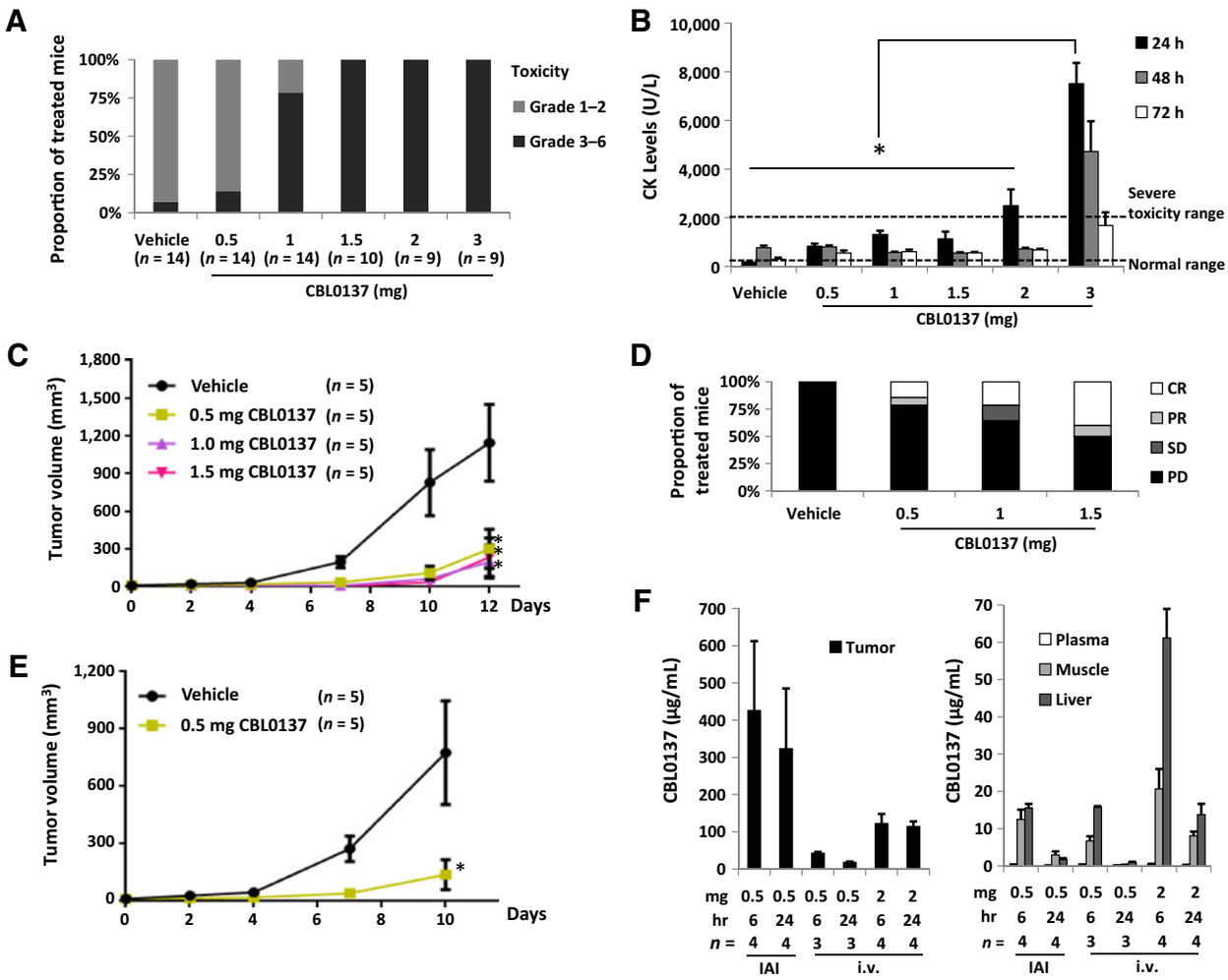

Figure 7.

IAI of CBL0137. **A,** toxicity after 15-minute IAI of vehicle (5% dextrose) or CBL0137 (0.5, 1, 1.5, 2, or 3 mg, pooled data). **B,** serum CK levels 24, 48, and 72 hours after IAI; dotted lines, range of toxicity (mean ± SEM; $n = 9$–14 mice/group; *, $P < 0.05$ by Student t test). **C,** representative tumor growth after 15-minute IAI of vehicle or CBL0137 (0.5, 1, or 1.5 mg; mean ± SEM; *, $P < 0.05$ by ANOVA, duplicate experiments). **D,** clinical responses after 15-minute IAI (pooled data). **E,** tumor growth after single 5-minute IAI of vehicle or 0.5 mg CBL0137 (mean ± SEM; *, $P < 0.05$ by ANOVA). **F,** tissue concentrations of CBL0137 collected 6 and 24 hours after 15-minute IAI or systemic (i.v., intravenous) CBL0137 injection (mean ± SEM; $n = 3$–4 mice/group, single experiment).

a consultant advisory board member for Incuron, Inc. No potential conflicts of interest were disclosed by the other authors.

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