

香港防癌會研究資助：2017 年度報告

研究標題：精氨酸酶和化療藥物聯合使用對鱗狀細胞肺癌的治療研究（體內與體外研究）

研究者及單位：

主要研究者：林詩鈞博士，哲學博士，博士後，香港大學內科學系。

合作研究者：何重文醫生，醫學博士，英國倫敦皇家內科醫學院榮授院士，臨床副教授，香港大學內科學系。

香港防癌會研究資助批准日期：2016 年 2 月 4 日

研究開始日期：2016 年 4 月 1 日

研究年期：1 年

報告和中期結果

我們在原本的研究提案中所列的研究目的包括以下的研究：

- 精氨酸酶在鱗狀細胞肺癌中的體外活性。
- 精氨酸酶和化療藥物（紫杉醇，吉西他濱和順鉑）在鱗狀細胞肺癌的協同效應（體外活性）。
- 精氨酸酶或與化療藥物組合對鱗狀細胞肺癌中的作用機制。
- 精氨酸酶或與化療藥物組合對鱗狀細胞肺癌裸鼠移植瘤的體內活性。

在過去一年，我們完成了鱗狀細胞肺癌細胞系體外活性和兩個鱗狀細胞肺癌裸鼠移植瘤的實驗。簡而言之，我們已經證實聚乙二醇化精氨酸酶 1（BCT-100）在鱗狀細胞肺癌細胞和其中一個裸鼠移植瘤具有良好的抗癌活性。以下是我們詳細的結果報告。

精氨酸酶在鱗狀細胞肺癌的體外活性

我們使用了 3 條鱗狀細胞肺癌細胞系（SK-MES-1，H520 和 H2170）。BCT-100 抑制鱗狀細胞肺癌細胞系的生長，通過噻唑藍（MTT）細胞活力測試所測定的 IC₅₀ 值分別為 13.7 ± 0.6 ， 14.0 ± 0.8 和 14.5 ± 0.3 毫單位/毫升（圖 1）。

精氨酸酶與化療藥物（紫杉醇，吉西他濱和順鉑）在鱗狀細胞肺癌的協同效應(體外)

我們研究了不同化學治療藥物（紫杉醇，吉西他濱和順鉑）與 BCT-100 的協同效應。然而，幾乎所有細胞系中的不同組合均沒有協同效應（圖 2）。最後，我們決定集中研究 BCT-100 單獨使用對鱗狀細胞肺癌的影響。

BCT-100 對細胞生長抑制的機制（體外）

精氨基琥珀酸合成酶（ASS1）和鳥氨酸轉氨甲醯酶（OTC）是尿素循環中的關鍵酶，負責補充細胞內精氨酸儲存。我們在所有細胞系中檢測不到精氨基琥珀酸合成酶和鳥氨酸轉氨甲醯酶的內源性表達（不顯示數據），表明鱗狀細胞肺癌對精氨酸酶的潛在敏感性。

對所有細胞系，BCT-100 均沒有改變 B 淋巴細胞瘤-2（Bcl-2）和生存素（survivin）的表達以及沒有把脫氧核糖核酸修復酶(PARP)和凋亡蛋白酶（caspase-3）切割，表明不涉及凋亡（不顯示數據）。此外，所有細胞系都不能檢測到磷化蛋白激酶 B（pAKT）和磷化細胞外調節蛋白激酶（pErk），也檢測不到 BCT-100 對磷化蛋白激酶 B 和磷化細胞外調節蛋白激酶的下調（數據未顯示），似乎 BCT-100 對體外細胞生長的抑制作用不是由細胞凋亡和對磷化蛋白激酶 B / 細胞外調節蛋白激酶途徑所導致。所以，體外機制仍然未明。

BCT-100 對鱗狀細胞肺癌裸鼠移植瘤的作用(體內)

我們成功建立兩個鱗狀細胞肺癌裸鼠移植瘤模型（以下簡稱“移植瘤模型”）（SK-MES-1 和 H520）。在不同組中裸鼠的最初腫瘤大小沒有顯著差異（不顯示數據）。圖 3 顯示了實驗期間對照組和 BCT-100 治療組中的相對腫瘤大小，BCT-100（60 毫克/公斤）抑制 SK-MES-1 移植瘤模型中的腫瘤生長，但不能抑制 H520 移植瘤模型的腫瘤生長。在不同組中裸鼠的體重並沒有差異證明 BCT-100 治療並沒有毒性（ $p > 0.05$ ）（不顯示數據）。

我們通過蛋白質印跡法研究精氨酸琥珀酸合成酶和鳥氨酸轉氨甲醯酶的內源性表達，精氨酸琥珀酸合成酶在 SK-MES-1 移植瘤模型中的表達高於 H520 移植瘤模型。兩種移植瘤模型的鳥氨酸轉氨甲醯酶水平均處於低水準（圖 4）。由於在 H520 移植瘤模型中精氨酸琥珀酸合成酶和鳥氨酸轉氨甲醯酶（尿素循環的耗盡的關鍵酶）表達都處於低水準，所以理論上它對 BCT-100 治療應當是敏感的。

內源性精氨酸酶 2（Arginase 2）在肺癌病人組織中的表達比較高，然而，精氨酸酶 2 不抑制免疫系統，也不影響疾病發展（Rotondo et al., 2008）。我們建議具有高內源性精氨酸酶 2 表達的移植瘤模型會比較適應低精氨酸水準，並且不會受 BCT-100 的影響。同時，我們發現在 H520 移植瘤模型中內源性精氨酸酶 2 有高水準表達（圖 5）。

PEG-BCT-100 可以在兩個移植瘤模型的治療組中的腫瘤樣品中發現（圖 6），同時把血清精氨酸濃度降低。另一方面，在 SK-MES-1 移植瘤模型中，只有 60 毫克/公斤 BCT-100 組可以降低腫瘤內精氨酸水準。在 H520 移植瘤模型的對照組中瘤內精氨酸的水準非常低，並且不能通過 BCT-100 進一步降低（圖 7），這可能解釋了 BCT-100 在該模型中的無效性。

在 SK-MES-1 移植瘤模型中，BCT-100 降低了周期蛋白 A2，周期蛋白 B1，周期蛋白 D3，周期蛋白 E1 和周期蛋白依賴性激酶 4 的表達，因此推斷細胞週期停滯在 G1 期，但這現象不在 H520 移植瘤模型中發生（圖 8）。此外，BCT-100 導致 SK-MES-1 移植瘤模型中的增殖因數 Ki67 的表達降低，這現象也不在 H520 移植瘤模型中發生。所以，BCT-100 只可以抑制 SK-MES-1 移植瘤模型中的腫瘤細胞增生（圖 9）。

我們在轉移酶介導的三磷酸脫氧鳥苷-生物素刻痕末端標記（TUNEL）測定中證明在 BCT-100 可以增加 SK-MES-1 移植瘤模型中的癌細胞凋亡，但並不在 H520 移植瘤模型中發生（圖 10）。

概要

BCT-100（聚乙二醇化精氨酸酶 1）抑制 SK-MES-1，H520 和 H2170 細胞以及 SK-MES-1 移植瘤模型的腫瘤生長。我們沒有觀察到 BCT-100 與鱗狀細胞肺癌中常用的化學治療劑的協同治療作用。在 H520 移植瘤模型中，精氨酸酶 2 的高內源性表達可能是 BCT-100 無效的原因。BCT-100 抑制 SK-MES-1 移植瘤模型的腫瘤生長可以透過精氨酸消耗引致細胞週期停滯在 G1 期和凋亡來解釋。

未來的計劃

- 1.我們將建立第三個移植瘤模型（新購買的鱗狀細胞肺癌細胞系）以進一步研究 BCT-100 在體內的作用。
- 2.當我們完成對第三個移植瘤模型的實驗後，我們將投稿文章。

參考文獻：

Rotondo R, Mastracci L, Piazza T, Barisione G, Fabbi M, Cassanello M, Costa R, Morandi B, Astigiano S, Cesario A, Sormani MP, Ferlazzo G, Grossi F, Ratto GB, Ferrini S, Frumento G. Arginase 2 is expressed by human lung cancer, but it neither induces immune suppression, nor affects disease progression. Int J Cancer. 2008, 123, 1108-16.

財務細目

截至 2017 年 2 月 20 日，支出如下：

	2016 年 9 月 12 日	2017 年 2 月 20 日	全年
項目	銀碼 (\$)	銀碼 (\$)	銀碼 (\$)
抗體	12583.6	43256.76	55840.36
蛋白質印跡	2886.7	9580.9	12467.6
細胞培養和消耗品	32466.84	22873.7	55340.54
試劑盒	50814.8	12916.8	63731.6
動物實驗	5350	7199.9	12549.9
大學開銷	17641	17641	35282
小計	121742.94	113469.06	235212

鱗狀細胞肺癌細胞系

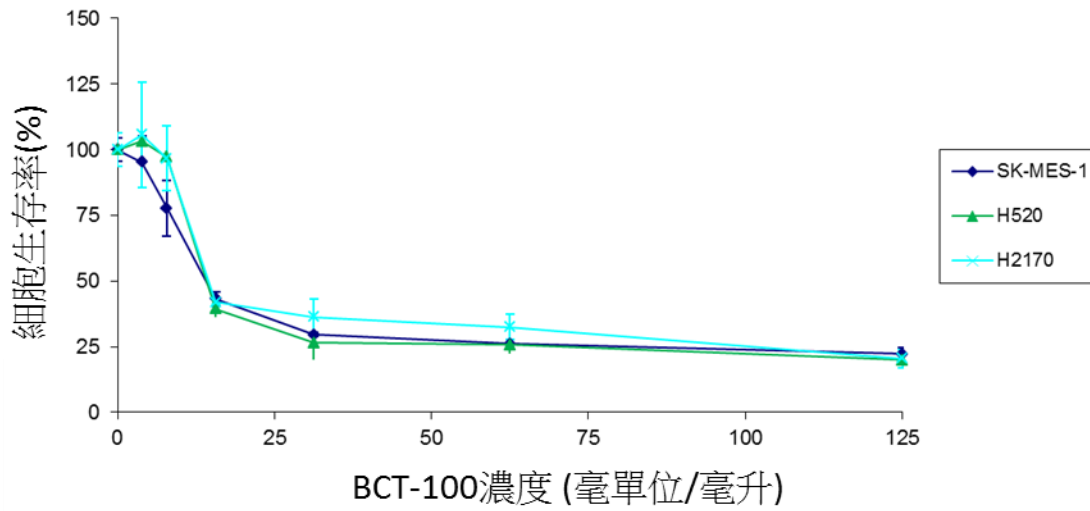
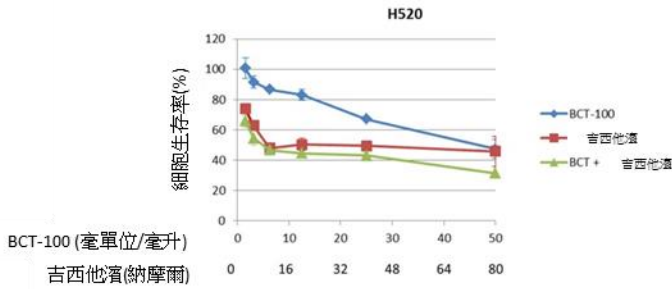
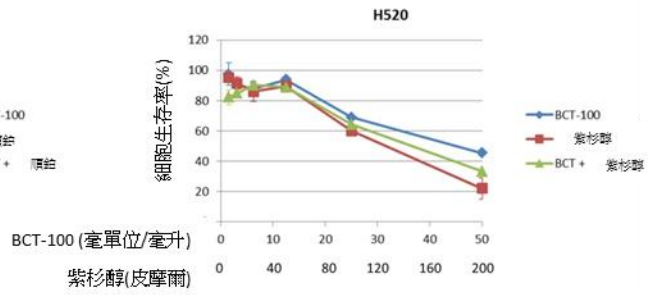
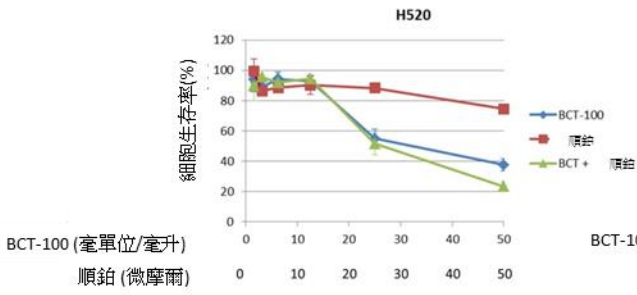
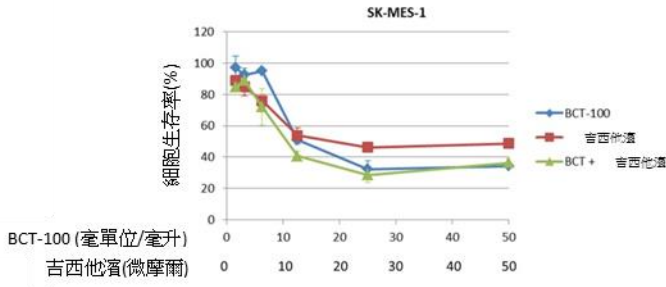
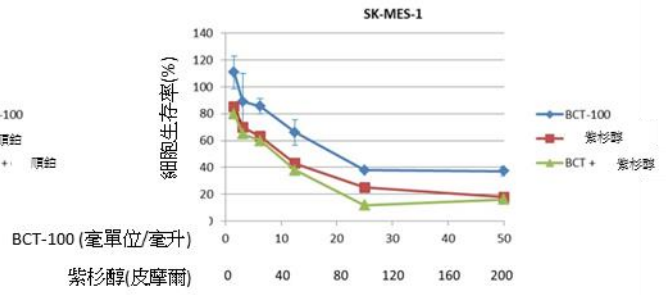
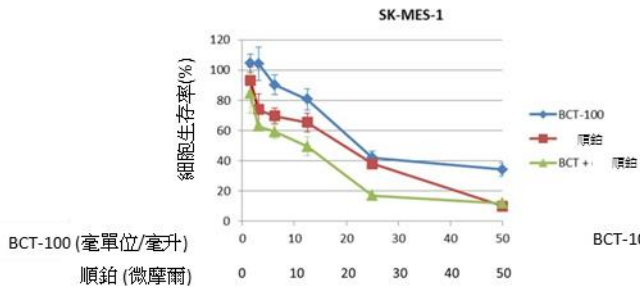


圖 1：BCT-100 處理後鱗狀細胞肺癌細胞系的生還率。BCT-100 可以抑制 SK-MES-1，H520 和 H2170 細胞的生長。



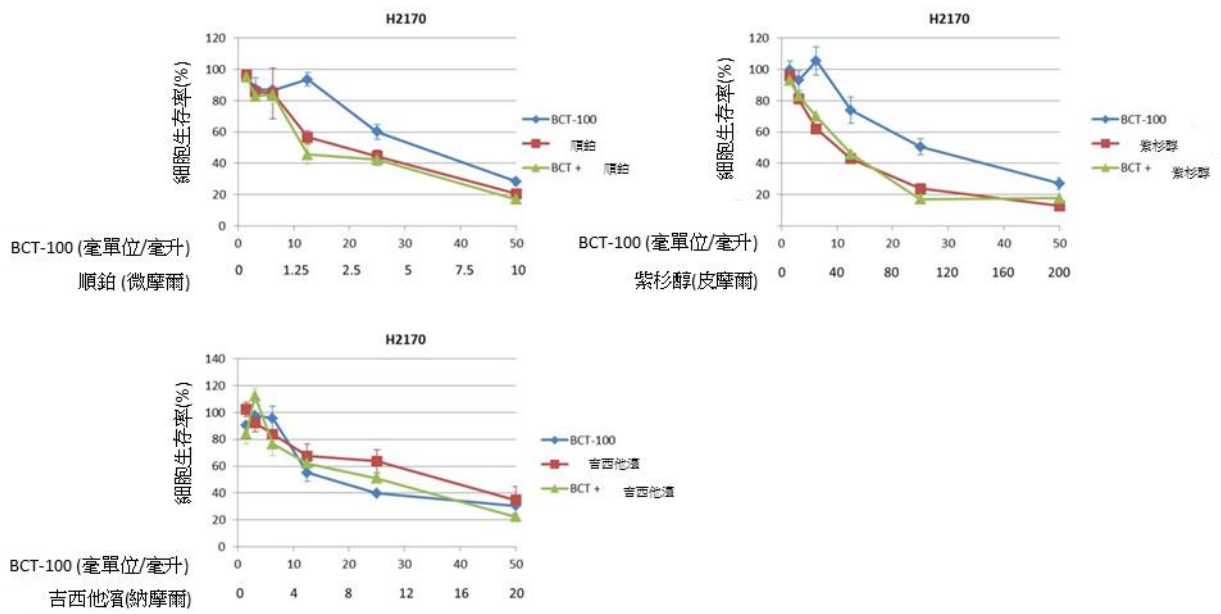
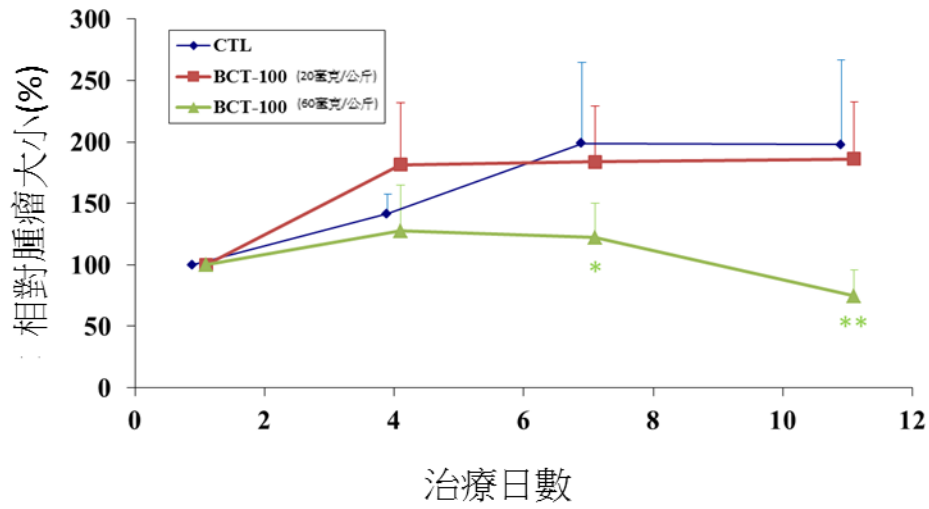


圖 2：BCT-100 與化療藥物（紫杉醇，吉西他濱和順鉑）在鱗狀細胞肺癌細胞系的體外組合效應。BCT-100 與紫杉醇，吉西他濱或順鉑組合對所有細胞系均沒有協同作用。

SK-MES-1移植瘤模型



H520移植瘤模型

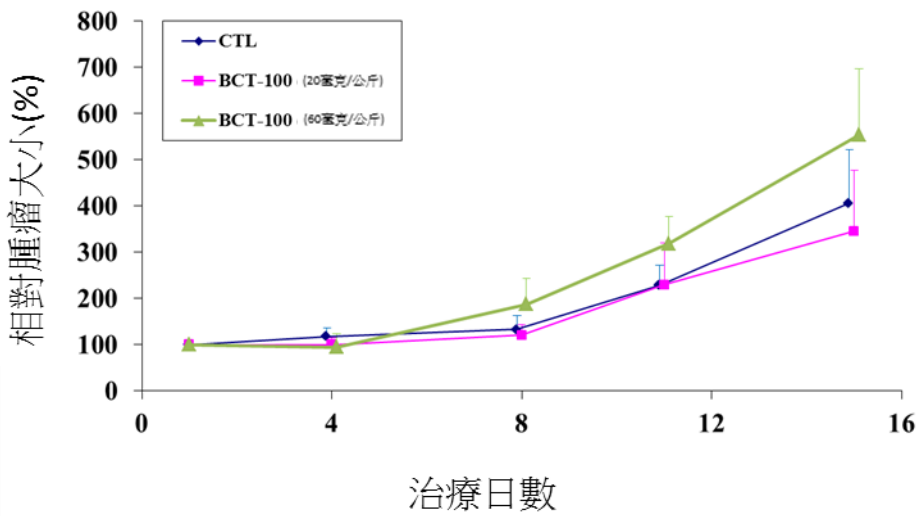


圖 3：SK-MES-1 和 H520 移植瘤模型的腫瘤在對照組和 BCT-100 治療組中的生長狀況。BCT-100 (60 毫克/公斤 kg) 抑制 SK-MES-1 移植瘤模型的腫瘤生長，但不能抑制 H520 移植瘤模型的腫瘤生長。p 值<0.05 定義為具有統計顯著性 (* : p < 0.05 , ** : p < 0.01) 。

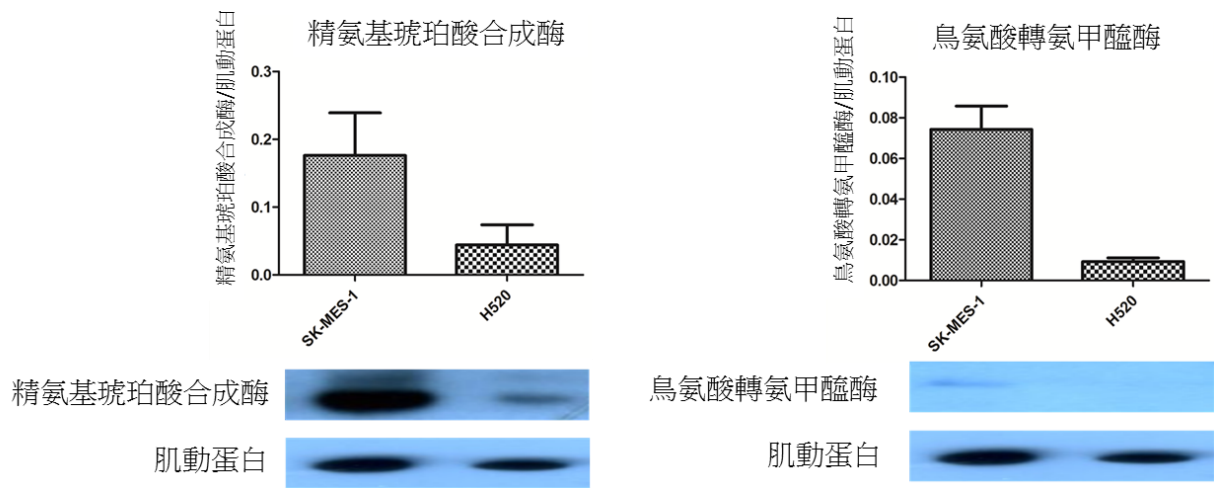


圖 4：SK-MES-1 和 H520 移植瘤模型的精氨基琥珀酸合成酶和鳥氨酸轉氨甲醯酶的內源性表達。精氨基琥珀酸合成酶在 SK-MES-1 移植瘤模型中內源性表達較高。鳥氨酸轉氨甲醯酶在兩種移植瘤模型的表達均弱。

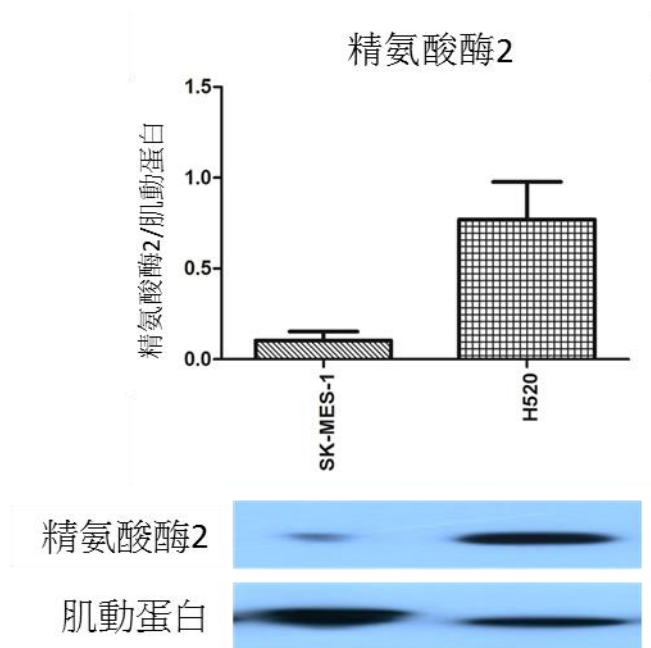


圖 5：內源性精氨酸酶 2 在 SK-MES-1 和 H520 移植瘤模型中的表達。內源性精氨酸酶 2 在 H520 移植瘤模型中表達很高。

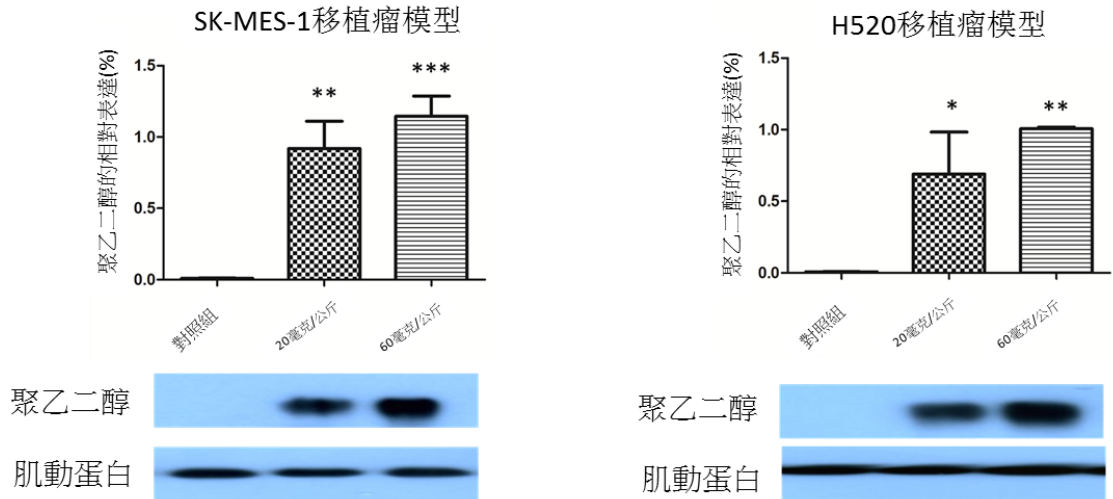


圖 6：SK-MES-1 和 H520 移植瘤模型的腫瘤內聚乙二醇化 BCT-100 水平。在兩個移植瘤模型的治療組中的腫瘤中可以檢測到聚乙二醇化 BCT-100。p 值<0.05 定義為具有統計學顯著性 (*：p <0.05，**：p <0.01，***：p <0.001)。

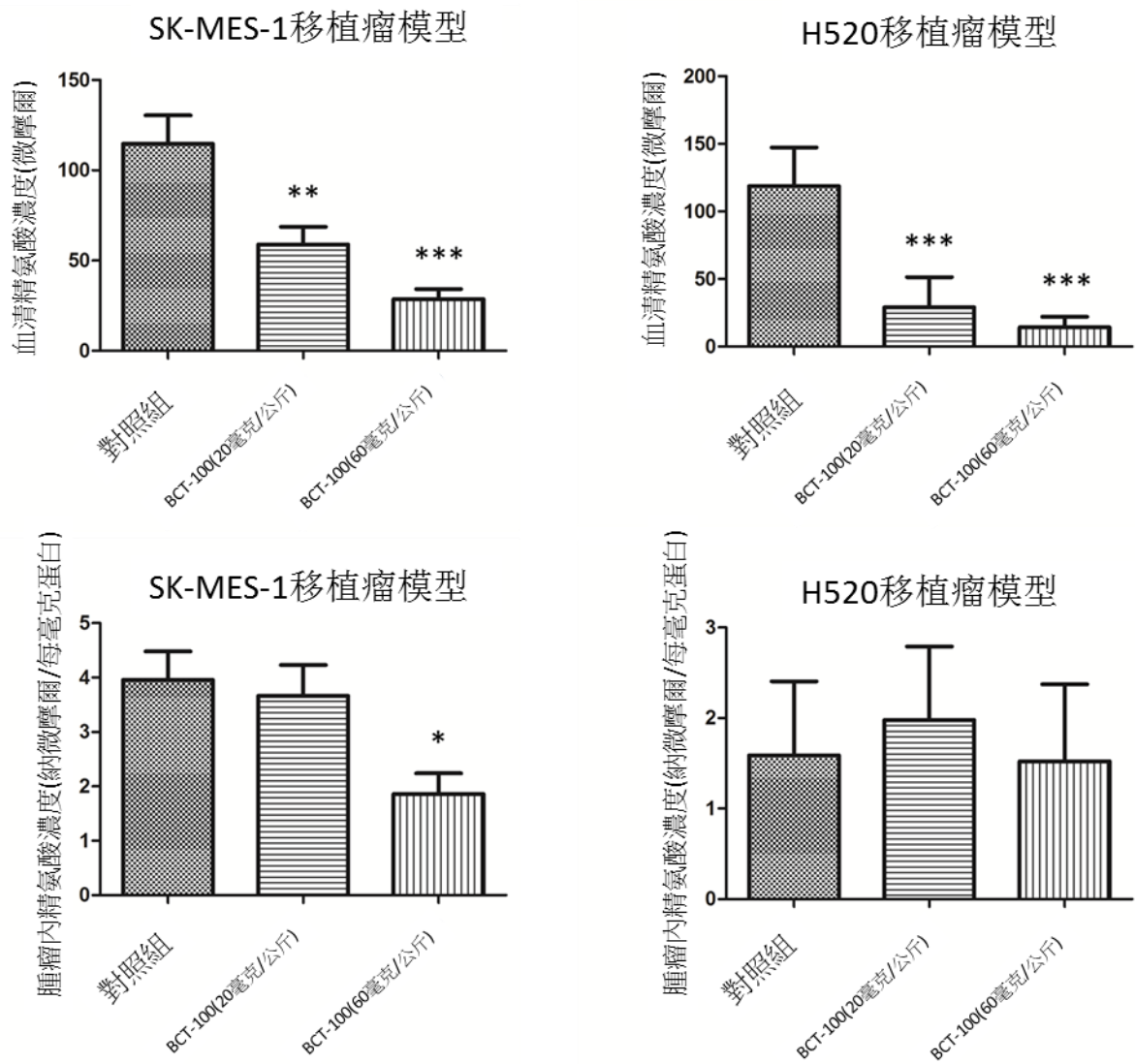


圖 7：在 SK-MES-1 和 H520 移植瘤模型的對照組和 BCT-100 治療組中的血清和腫瘤內精氨酸水平。BCT-100 可以降低兩個移植瘤模型中的血清精氨酸濃度和 SK-MES-1 移植瘤模型中的腫瘤內精氨酸水平。p 值 < 0.05 定義為具有統計學顯著性 (* : p < 0.05, ** : p < 0.01, *** : p < 0.001)。

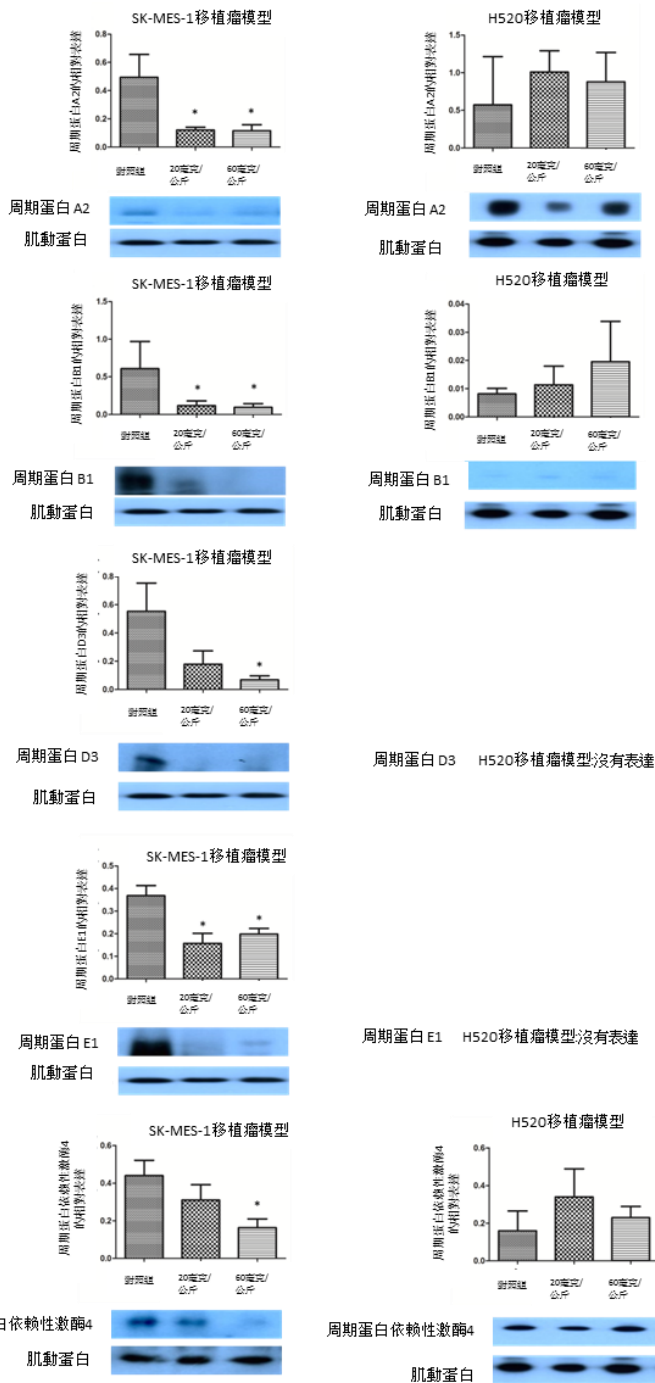


圖 8：周期蛋白 A2，周期蛋白 B1，周期蛋白 D3，周期蛋白 E1 和周期蛋白依賴性激酶 4 在 SK-MES-1 和 H520 移植瘤模型的對照組和 BCT-100 治療組中的表達。BCT-100 下調 SK-MES-1 移植瘤模型的周期蛋白 A2，周期蛋白 B1，周期蛋白 D3，周期蛋白 E1 和周期蛋白依賴性激酶 4 的表達，但這情況不在 H520 移植瘤模型中發生。p 值<0.05 定義為具有統計顯著性 (*：p < 0.05)。

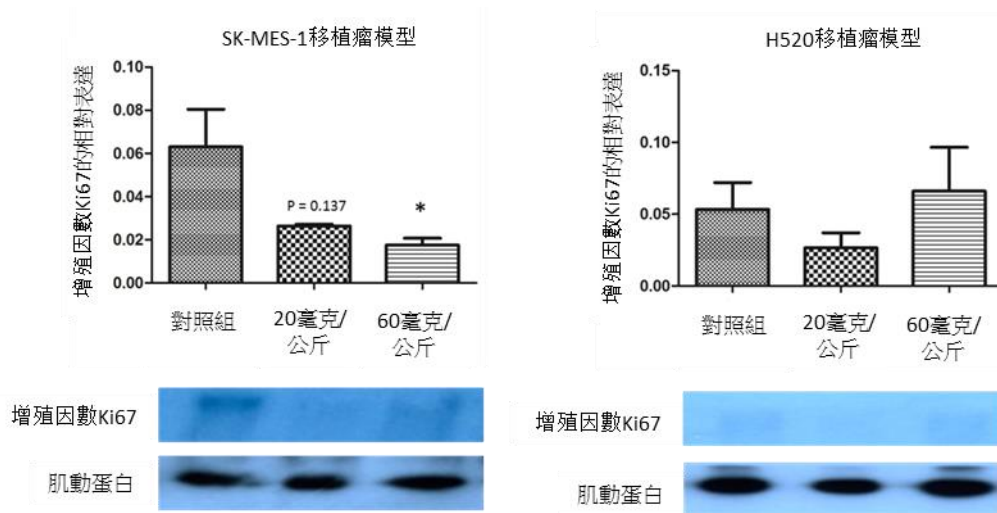
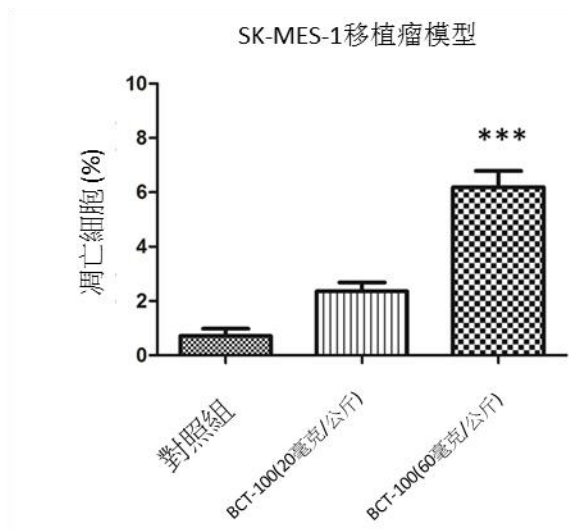
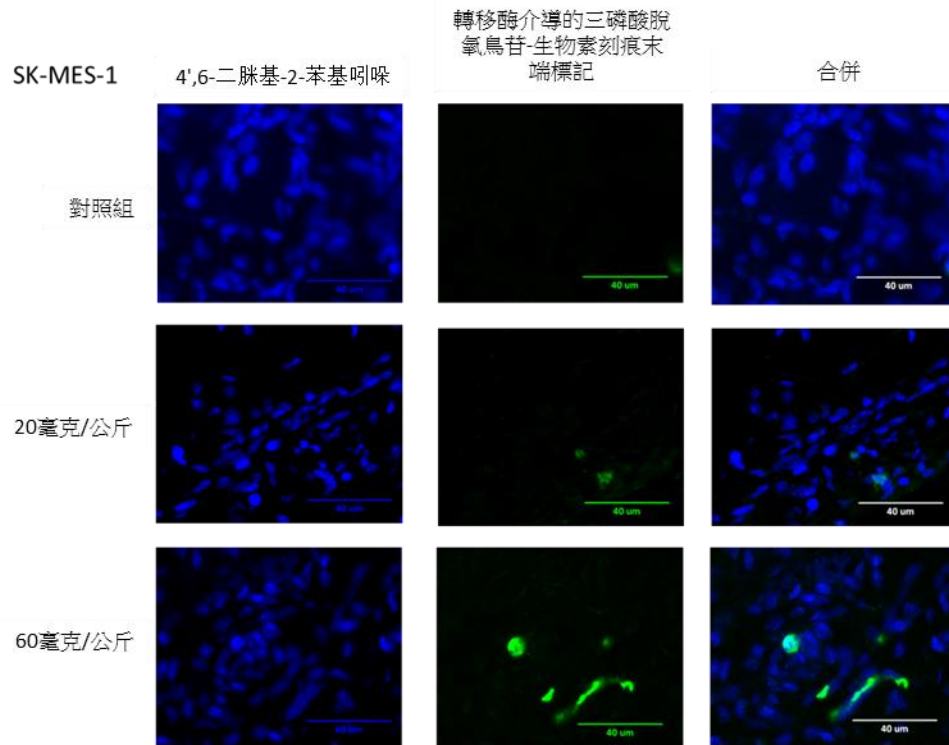


圖 9：增殖因數 Ki67 在 SK-MES-1 和 H520 移植瘤模型對照組和 BCT-100 治療組的表達。BCT-100 所抑制 SK-MES-1 移植瘤模型的 Ki67 的表達，但不能抑制 H520 移植瘤模型的 Ki67 的表達。p 值<0.05 定義為具有統計學顯著性 (* : p < 0.05)。



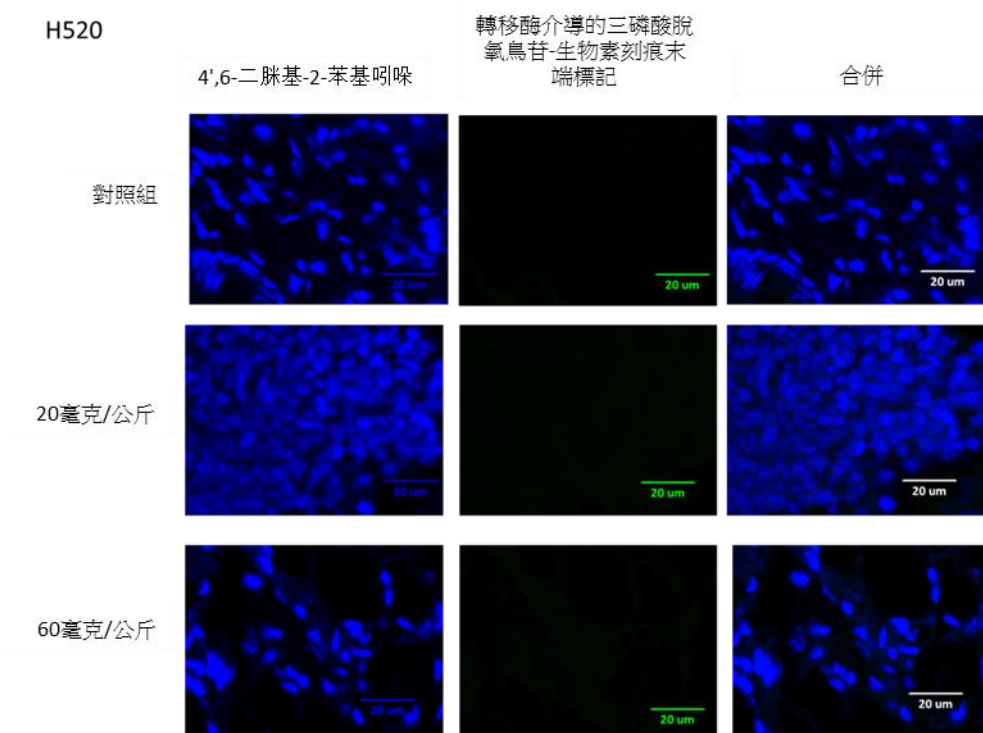


圖 10：BCT-100 引起移植瘤模型中的癌細胞進行凋亡。60 毫克/公斤 BCT-100 可引致 SK-MES-1 移植瘤模型的癌細胞凋亡，但這情況不在 H520 移植瘤模型發生。p 值 <0.05 定義為具有統計學顯著性 (***)：p <0.001)。

The Hong Kong Anti-Cancer Society research grant: final report 2017

Study title: In vitro and in vivo combination of arginase and chemotherapeutic drugs in squamous cell lung carcinoma

Investigators and affiliations:

Principal investigator: Dr. Sze-Kwan LAM PhD, Post-doctoral Fellow, Department of Medicine, The University of Hong Kong

Co-investigator: Dr. James Chung-Man HO M.D. FRCP, Clinical Associate Professor, Department of Medicine, The University of Hong Kong

HKACS research grant approval date: 4 February 2016

Project start date: 1 April 2016

Study duration: 1 year

Final report and interim results:

The aims of our study, as listed in the original study proposal, would include the following:

1. To explore the *in vitro* activity of arginase in SCC.
2. To investigate the possible *in vitro* combination effect between arginase and chemotherapeutic drugs (paclitaxel, gemcitabine and cisplatin) in SCC.
3. To examine the mechanisms of action of arginase and/or chemotherapeutic drugs in SCC.
4. To study the *in vivo* activity of arginase and/or chemotherapeutic drugs in SCC xenograft models.

Over the past year, we have finished the experiments for the cell line model and two nude mice xenograft models. In brief, we have confirmed activity of pegylated arginase 1 (BCT-100) in SCC cell lines and one of xenograft models. The more detail report of our results is as follows:

In vitro activity of arginase in SCC

A panel of 3 SCC cell lines (SK-MES-1, H520 and H2170) was used. BCT-100 reduced the viability of SCC cell lines with the IC_{50} values were obtained by MTT cell viability assay (13.7 ± 0.6 , 14.0 ± 0.8 and 14.5 ± 0.3 mU/ml respectively) (Figure 1).

In vitro combination effect between arginase and chemotherapeutic drugs (paclitaxel, gemcitabine and cisplatin) in SCC

The combination effect of different chemotherapeutic drugs (paclitaxel, gemcitabine and cisplatin) with BCT-100 was studied. However, there was no synergistic effect in all combinations in almost all cell lines (Figure 2). Finally, the effect of BCT-100 alone on SCC was focused in this study.

Mechanism of cell growth inhibition *in vitro*

Argininosuccinate synthetase (ASS1) and ornithine transcarbamylase (OTC) are key enzymes in urea cycle, responsible for replenishing intracellular store of arginine. They were not expressed in all cell lines in our experiments (data not shown), indicating potential sensitivity to arginase, i.e. arginine depletion.

There was no alteration in expression of Bcl-2 and survivin as well as no cleavage of PARP and caspase-3 in all cell lines treated with BCT-100 (data not shown) indicating that apoptosis was not involved. Furthermore, pAKT and pErk were not detectable in control group, so no additional downregulation of pAKT and pERK could be demonstrated after BCT-100 treatment (data not shown). It seems that cell growth inhibition was not mediated by apoptotic and AKT/Erk pathways *in vitro*. The *in vitro* mechanism remained unknown.

In vivo effect of BCT-100 on tumor xenografts

Two xenograft models were successfully developed: SK-MES-1 and H520. There was no significant difference in baseline tumor size in mice amongst different groups (data not shown). The relative tumor size in the control and BCT-100 treatment arm during experiment was revealed in Figure 3. BCT-100 (60 mg/kg) suppressed tumor growth in SK-MES-1 xenograft model but not H520 xenograft model (Figure 3). There were no toxic effects upon BCT-100 treatment as evidenced from similar body weight in different groups ($p > 0.05$) (data not shown).

Basal expression of ASS1 and OTC was investigated by Western blot. ASS1 was highly expressed in SK-MES-1 xenograft when compared with H520 xenograft. OTC level was low in both xenografts (Figure 4). Since both OTC and ASS1 were under-expressed (i.e. depleted key enzymes of urea cycle) in H520 xenograft, it should be theoretically sensitive to BCT-100 treatment.

It has been reported that arginase 2 (ARG2) is expressed in human lung cancer, however, ARG2 does not induce immune suppression nor affect disease progression (Rotondo et al., 2008). We proposed SCC xenograft with high basal ARG2 expression would adapt low intratumoral arginine level and would not be affected by arginine depletion therapy. We found that ARG2 was highly expressed in H520 xenograft (Figure 5).

PEG-BCT-100 could be found in the tumor samples in both treatment arms in both xenografts (Figure 6) accompanied with decrease in serum arginine concentration. On the other hand, the

intratumoral arginine level could only be decreased in 60 mg/kg arm in SK-MES-1 xenograft. The intratumoral arginine level in control arm of H520 xenograft was very low and could not be further decreased by BCT-100 (Figure 7) which might account for the inefficacy of BCT-100 in this model.

Downregulation of cyclin A2, cyclin B1, cyclin D3, cyclin E1 and cdk4 by BCT-100 in SK-MES-1 xenograft indicating G1 arrest was mainly induced by BCT-100, but not in H520 xenograft (Figure 8). Furthermore, expression of proliferation factor, Ki67, was decreased in BCT-100 arm in SK-MES-1, but not H520 xenograft: proliferation of tumor cells in SK-MES-1 xenograft was inhibited (Figure 9).

Apoptotic cells were increased upon BCT-100 treatment as demonstrated in TUNEL assay in SK-MES-1 xenograft, but not H520 xenograft (Figure 10).

Summary

BCT-100 (pegylated arginase 1) suppressed tumor growth in SK-MES-1, H520 and H2170 cells as well as SK-MES-1 xenograft. There was no observed synergistic interaction of BCT-100 with commonly used chemotherapeutic agents in SCC. The lack of efficacy of BCT-100 in H520 xenograft might be due to high basal expression of arginase 2. The mechanisms of tumor suppression in SK-MES-1 xenograft were partially induced by G1 arrest and apoptosis, mediated by arginine depletion.

Future plan

1. One more xenograft model (new bought SCC cell line) will be established to further study the effect of BCT-100 *in vivo*.
2. Manuscript will be submitted for publication after completion of experiments on the additional xenograft.

References:

Rotondo R, Mastracci L, Piazza T, Barisione G, Fabbi M, Cassanello M, Costa R, Morandi B, Astigiano S, Cesario A, Sormani MP, Ferlazzo G, Grossi F, Ratto GB, Ferrini S, Frumento G. Arginase 2 is expressed by human lung cancer, but it neither induces immune suppression, nor affects disease progression. *Int J Cancer*. 2008, 123, 1108-16.

Financial breakdown

As of 20 Feb 2017, the expenditure is as follows:

	12 Sept 2016	20 Feb 2017	Whole Year
Items	Amount (\$)	Amount (\$)	Amount (\$)
Antibodies	12583.6	43256.76	55840.36
Western Blot	2886.7	9580.9	12467.6
Cell Culture and Consumable	32466.84	22873.7	55340.54
Assay	50814.8	12916.8	63731.6
Animal Work	5350	7199.9	12549.9
University Overhead	17641	17641	35282
Sub-Total	121742.94	113469.06	235212

Squamous cell lung carcinoma cell lines

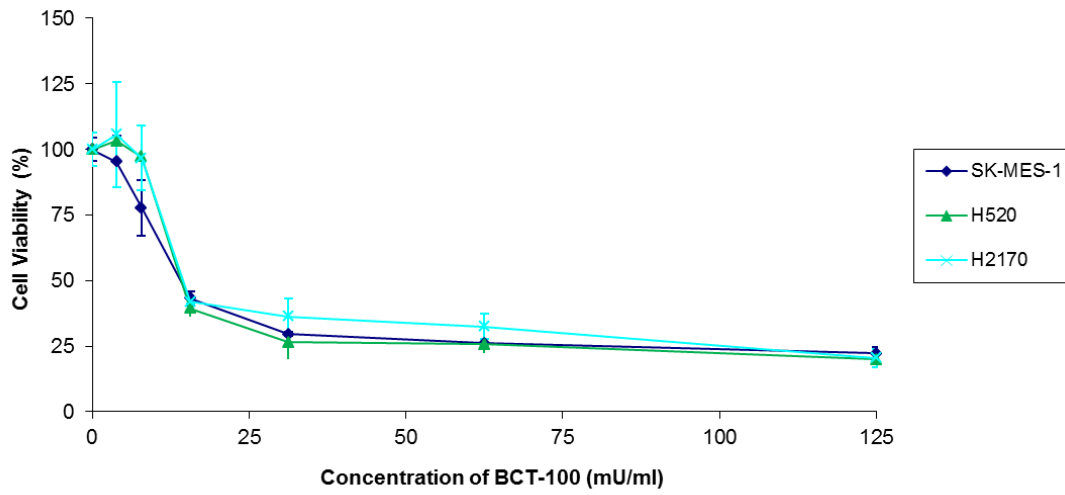
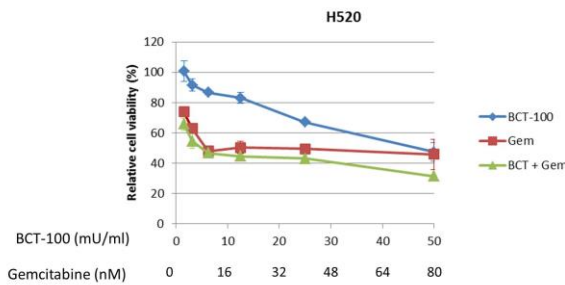
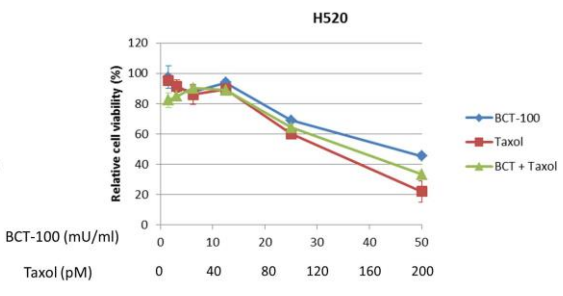
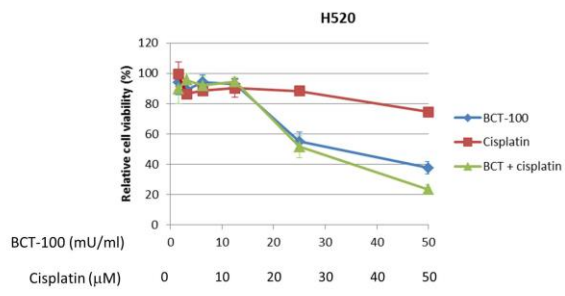
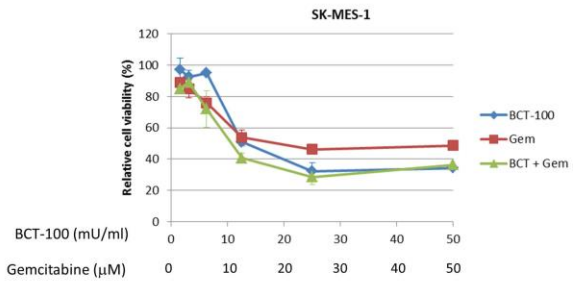
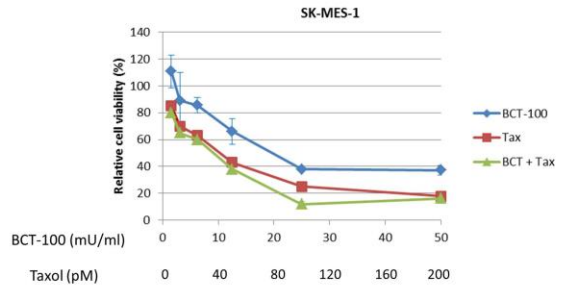
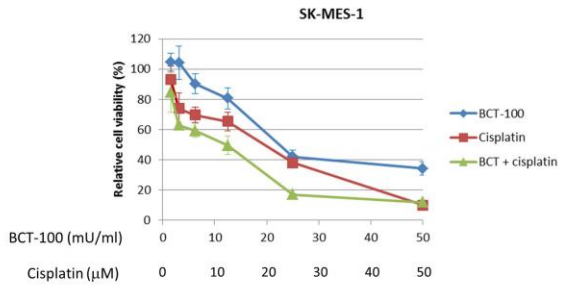


Figure 1: Cell viability of SCC cell lines upon BCT-100 treatment. Cell viability was reduced by treatment with BCT-100 in SK-MES-1, H520 and H2170 cells.



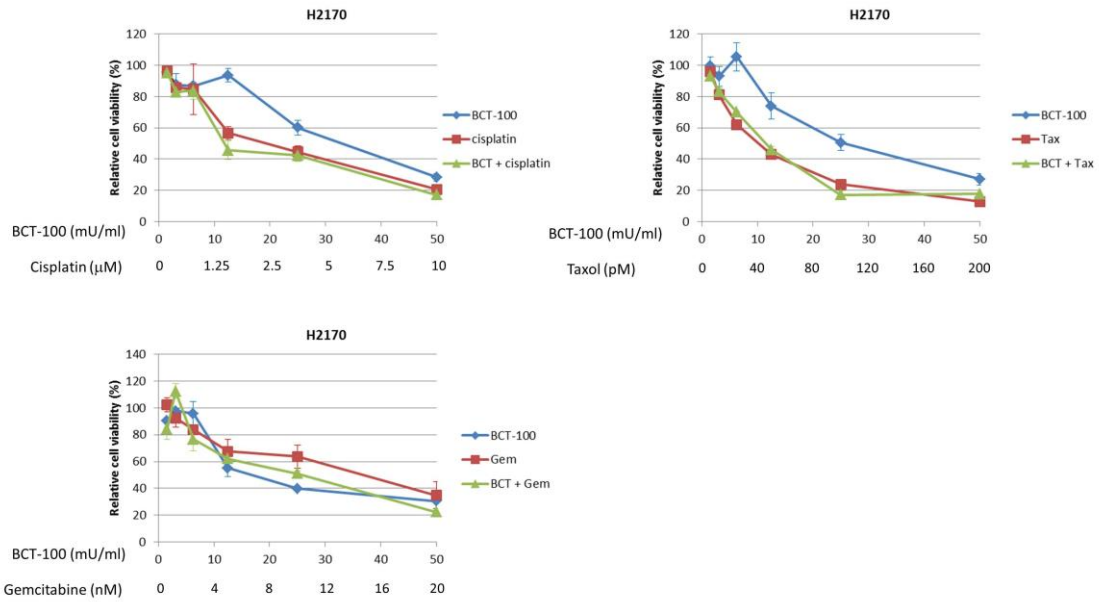


Figure 2: *In vitro* combination effect between BCT-100 and chemotherapeutic drugs (paclitaxel, gemcitabine and cisplatin) in SCC cell lines. No synergistic effect when combining BCT-100 with paclitaxel, gemcitabine or cisplatin in all cell lines.

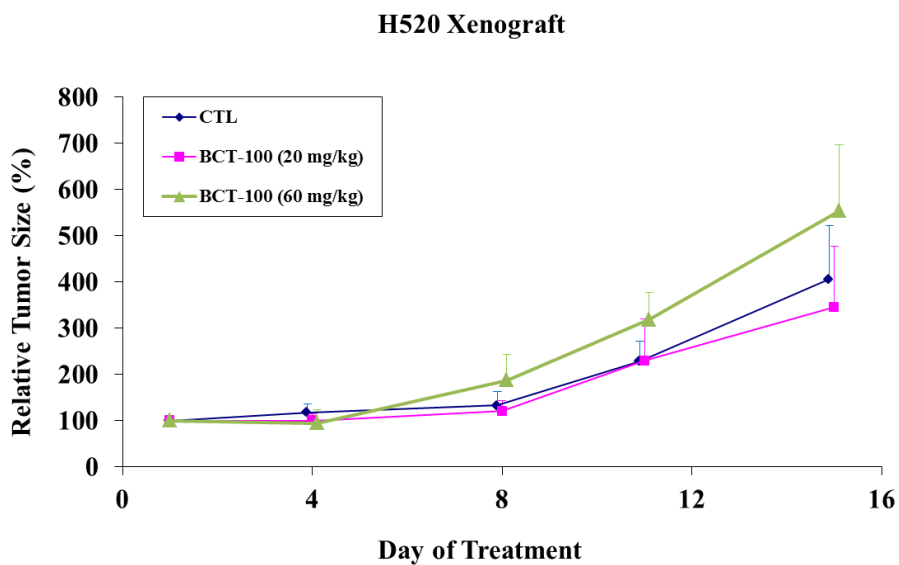
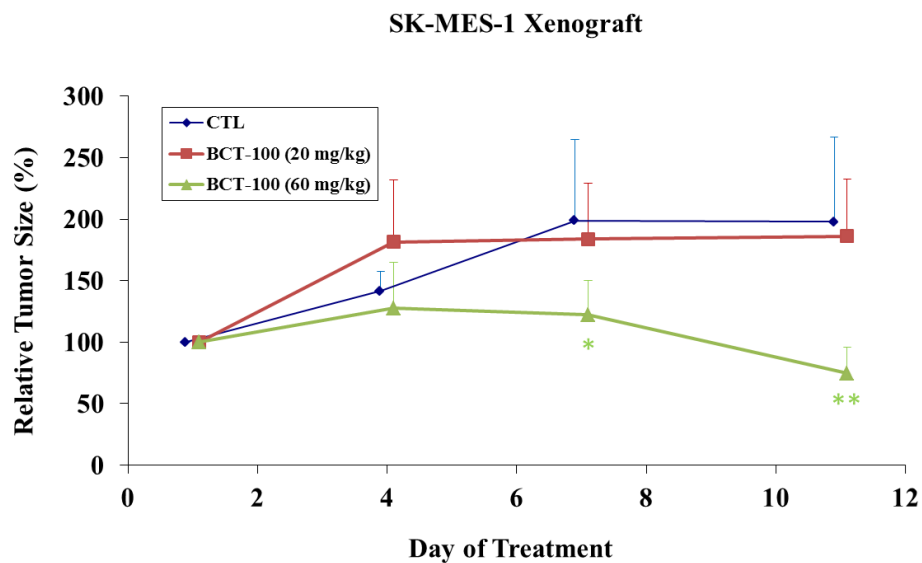


Figure 3. The relative tumor size in SK-MES-1 and H520 xenograft nude mice models in control and BCT-100 treatment arms. BCT-100 (60 mg/kg) suppressed tumor growth in SK-MES-1, but not H520 xenograft. A p-value < 0.05 defined statistical significance (*: p<0.05, **: p<0.01).

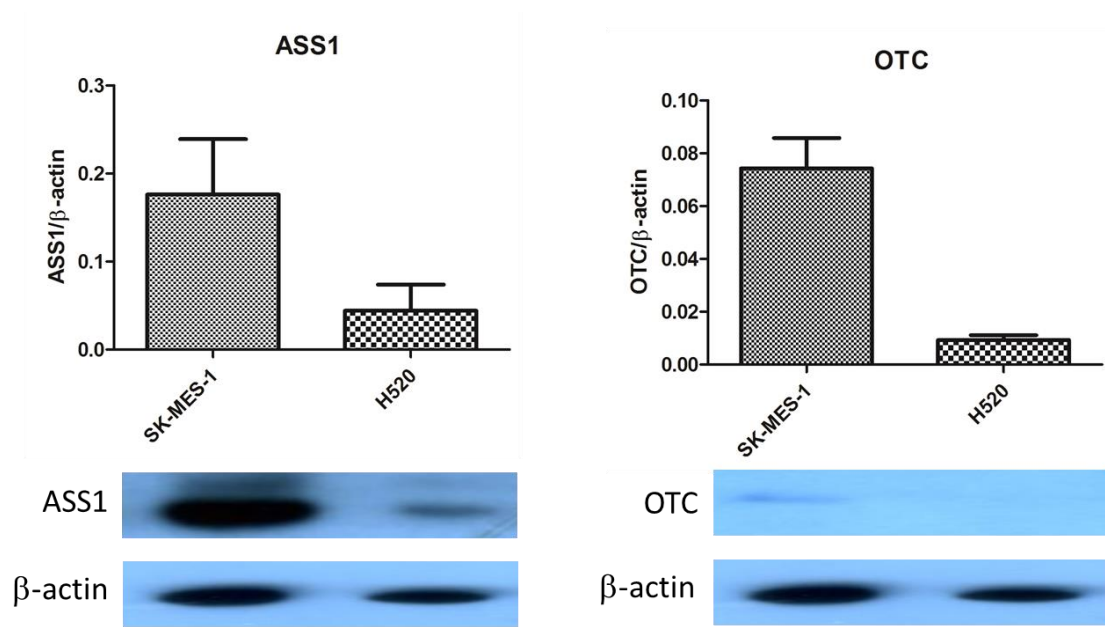


Figure 4. ASS1 and OTC expression in SK-MES-1 and H520 xenograft. ASS1 was highly expressed in SK-MES-1 xenograft. OTC was weakly expressed in both xenografts.

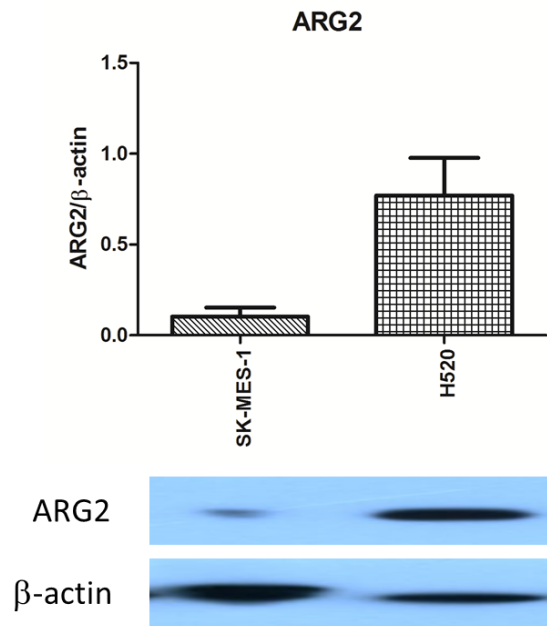


Figure 5. Basal arginase 2 (ARG2) expression in SK-MES-1 and H520 xenografts. Arginase 2 was highly expressed in H520 xenograft.

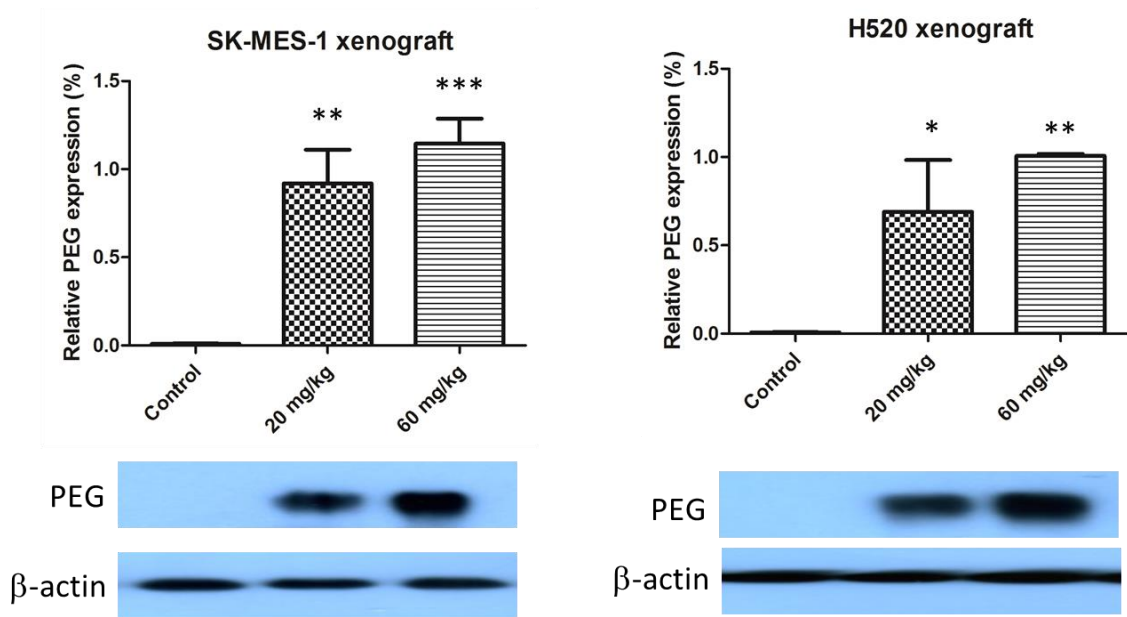


Figure 6. The intratumoral PEG-BCT-100 level in SK-MES-1 and H520 xenografts. PEG-BCT-100 could be found in the tumor in treatment arms of both xenografts. A p-value < 0.05 defined statistical significance (*: p<0.05, **: p<0.01, ***: p<0.001).

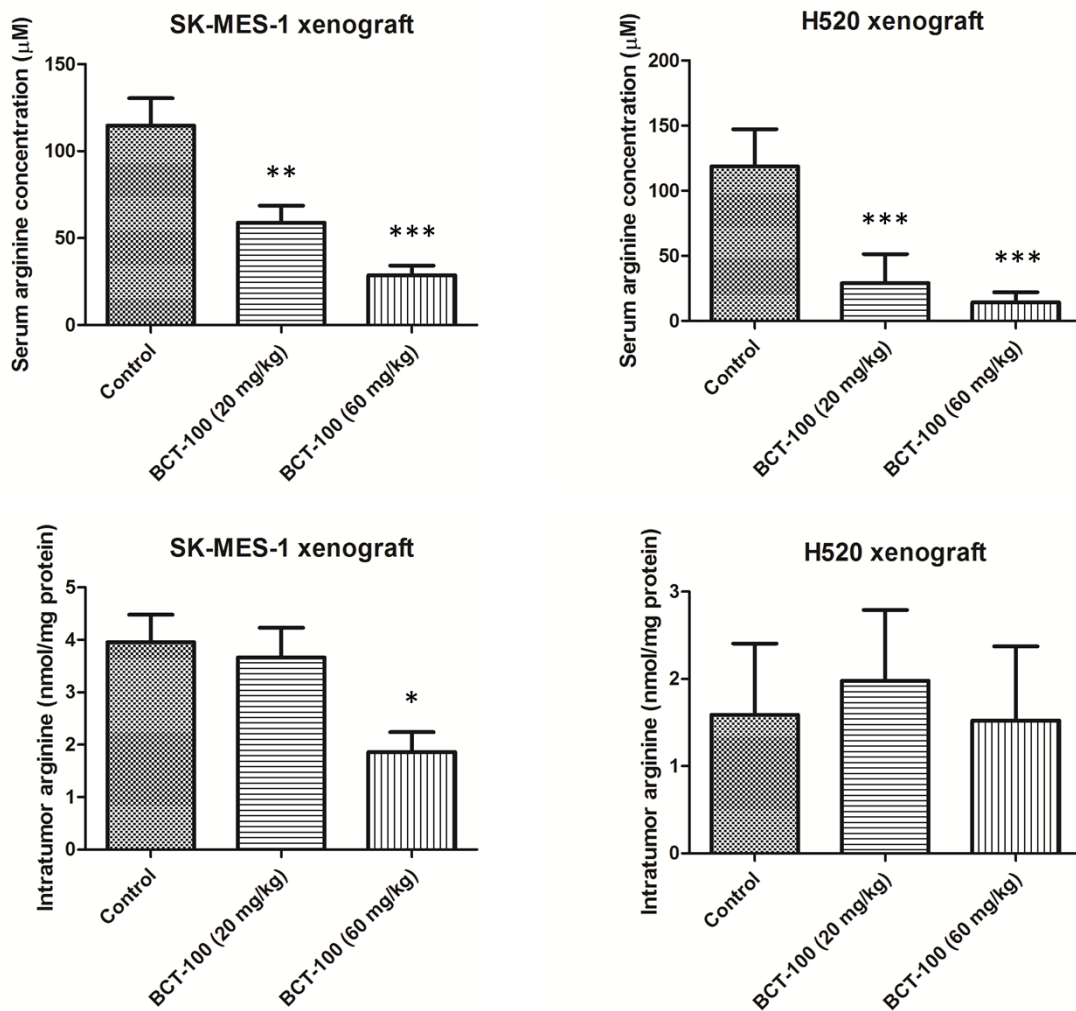


Figure 7. The serum and intratumoral arginine in control and BCT-100 treatment arms of SK-MES-1 and H520 xenografts. BCT-100 decreased serum arginine concentration in both xenograft while declined intratumoral arginine level in SK-MES-1 xenograft only. A p-value < 0.05 defined statistical significance (*: p<0.05, **: p<0.01, ***: p<0.001).

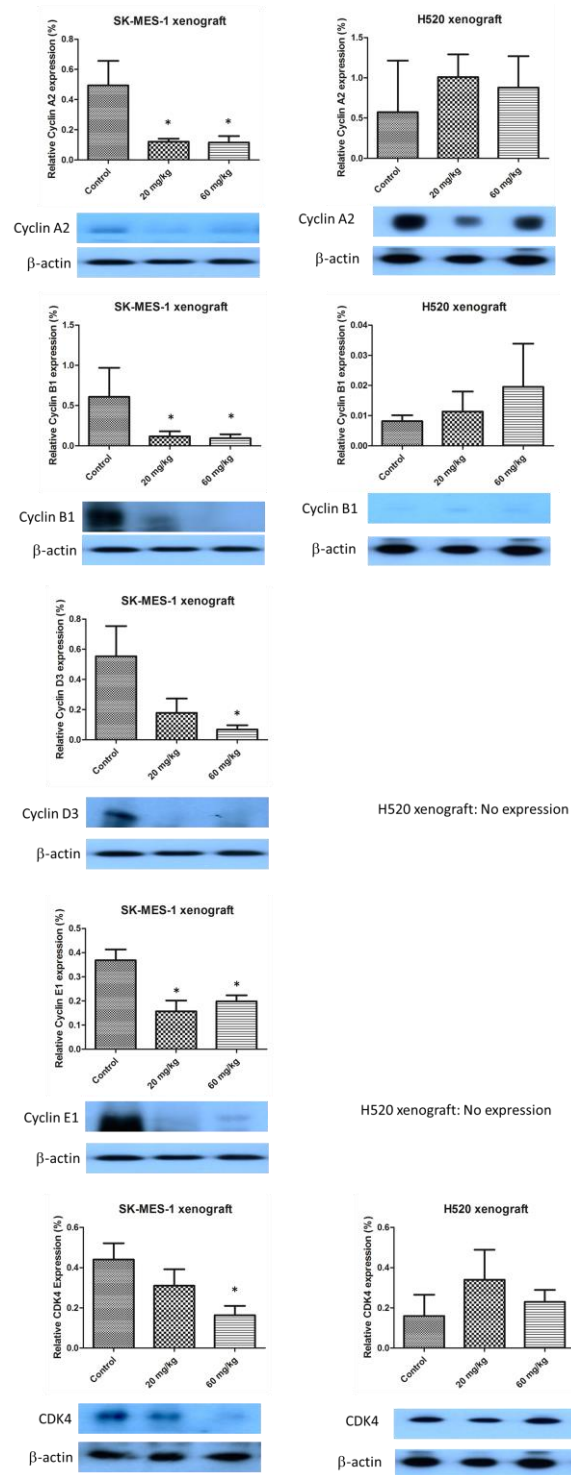


Figure 8. Expression of cyclin A2, cyclin B1, cyclin D3, cyclin E1 and CDK4 in control and BCT-100 treatment arms of SK-MES-1 and H520 xenografts. BCT-100 downregulated expression of cyclin A2, cyclin B1, cyclin D3, cyclin E1 and CDK4 in SK-MES-1 xenograft, but not H520 xenograft. A p-value < 0.05 defined statistical significance (*: p<0.05).

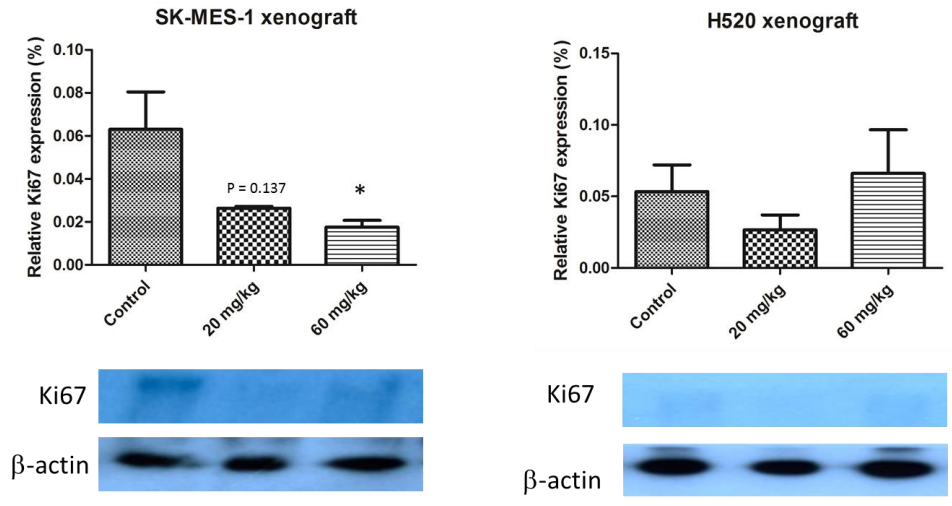
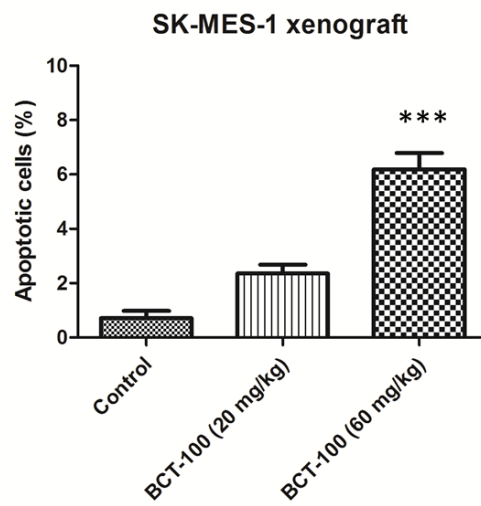
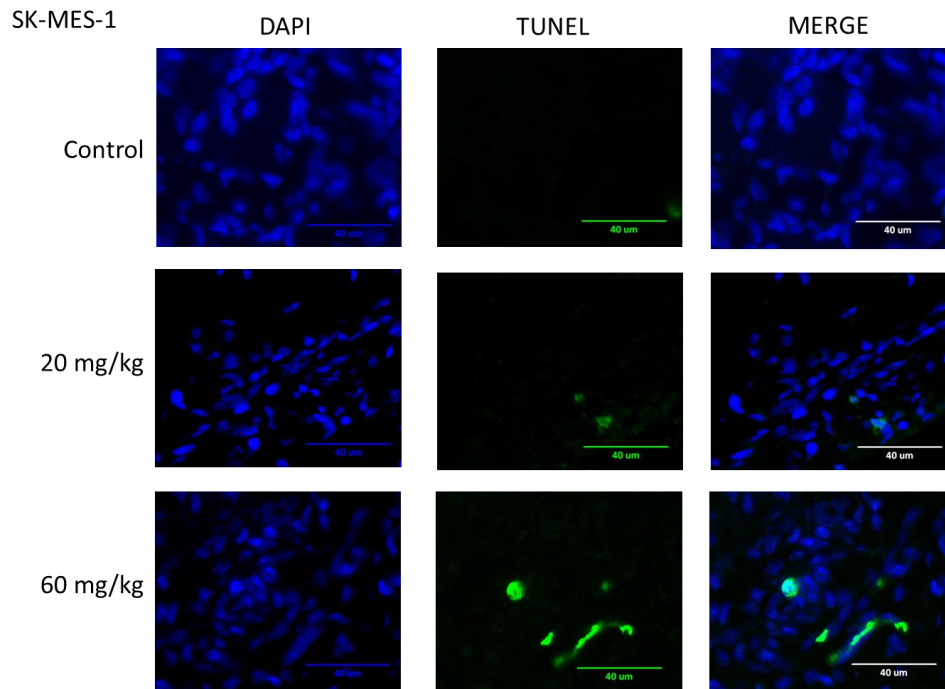


Figure 9. The expression of proliferation factor, Ki67, in control and BCT-100 treatment arms of SK-MES-1 and H520 xenografts. Expression of Ki67 was suppressed by BCT-100 in SK-MES-1 xenograft, but not H520 xenograft. A p-value < 0.05 defined statistical significance (*: p<0.05).



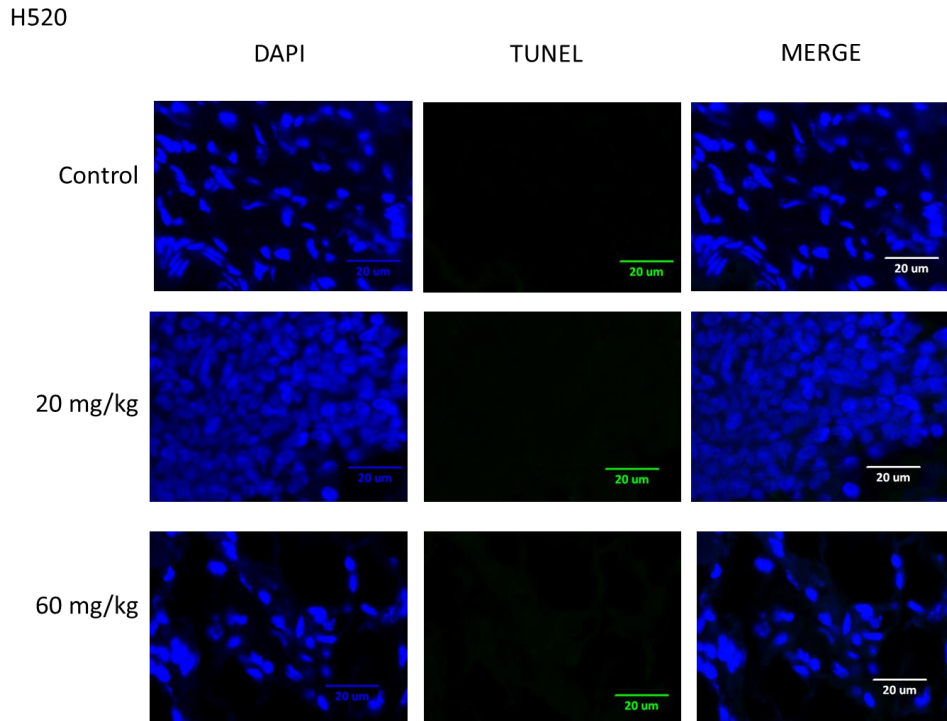


Figure 10. Apoptosis induction by BCT-100 in xenograft models. Apoptosis was induced by 60 mg/kg BCT-100 treatment arm in SK-MES-1, but not H520 xenograft. A p-value < 0.05 defined statistical significance (***: p<0.001).