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微生物免疫
與生物藥學系

探討*Aspergillus japonicas* β-fructofuranosidase轉換蔗糖效能與工業化製程

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摘要

近年來預防保健意識抬頭，各國開始發展保健食品，包括對於腸道微生物的正常菌相維持所需的益生源需求也持續增加，加上低熱量或無脂肪食品的市場更是日益增大，而菊粉以及果寡糖就是滿足這些條件的新型膳食纖維。果寡糖作為益生源，可促進腸道中的比菲德氏菌增長，抑制腸道壞菌生長，可調節腸道健康，同時比菲德氏菌也可鞏固口腔的衛生。比菲德氏菌的增加也可促進免疫系統的活化，增加免疫細胞的數量。果寡糖為一種水溶性膳食纖維無法直接被人體吸收，因此不會產生過多熱量，可降低血液中的游離脂肪、膽固醇，也不會增加血糖濃度，達到降血脂和降血糖的作用。近年來有許多文獻開始探討生產果寡糖的酵素以及大量果寡糖生產酶方法，但工業生產遭遇菌株無法長時間保存，且純化不易的問題。實驗室已知菌株 *Aspergillus japonicas*以蔗糖醋酸鈉溶液作為基質反應液，與酵素液反應轉換蔗糖，用以測試酵素液的活性以及比活性。利用實驗室級的超音波進行小量破菌，用以酵素位置的確認，以及酵素純化；並利用將反應過後的蔗糖溶液稀釋進行 HPLC 分析蔗糖轉換效率，以及醣類(果寡糖、蔗糖、葡萄糖、果 糖)比例分析。使用試量產級高壓均質器以及電動式高壓奈米粉碎機評估是否符合大量生產時的破菌設備。結果顯示，酵素多在菌體的細胞膜或菌體中，進行破壁離心後可提高酵素活性，也獲較佳的酵素純化條件。

材料方法

一、超音波破菌(50 mL小量破菌)
震盪 (400W) 20分鐘 (震盪15秒後停止15秒)，進行破菌。

二、高壓均質機破菌(20 L大量破菌)
以100 mpa 壓力進行破菌

三、蛋白質濃度檢測
使用Protein assay reagent（BIO-RAD），藉由已知的蛋白質溶液濃度測定酵素液中的可溶性蛋白含量。

四、硫銨沉澱法
利用硫酸銨兩水分子親和力較佳的特性，使不同疏水程度的蛋白質分別沉澱。用以分離酵素及濃縮蛋白。

五、HPLC分析
使用管柱Waters sugar pack-I分析反應過後各種醣類的比例以及濃度。

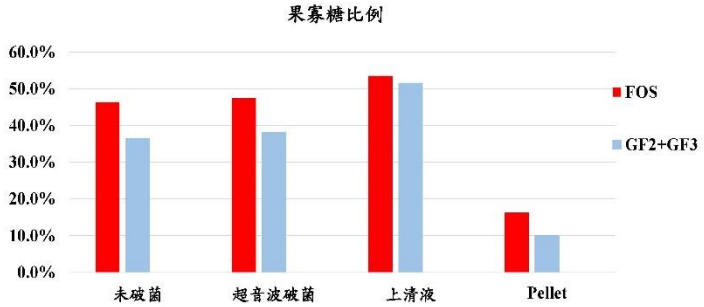
結果

表一、菌體胞內外酵素確認

	FOS	GF2+GF3
冷凍菌塊 未破菌	46.3%	36.5%
冷凍菌塊 未破菌上清液	19%	16%

表二、菌體破壁酵素活性

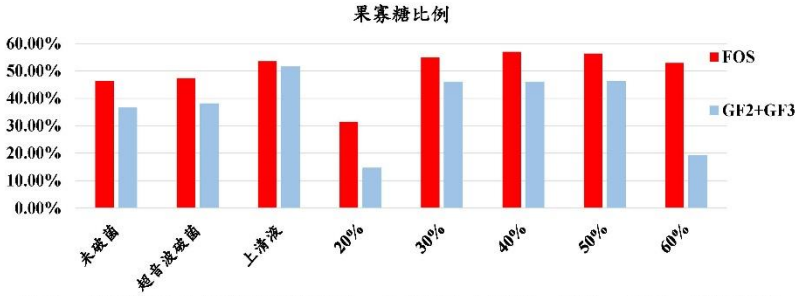
	酵素活性 (IU)	可溶性蛋白 (mg/g菌體)	回收率	酵素比活性 (IU/mg 蛋白)
冷凍菌塊 未破菌	5125	9.99	100%	513
冷凍菌塊 超音波破菌	5125	16.49	100%	310
破菌後離心上清液	3875	2.34	75.6%	1656
破菌後離後pellet	100	14.3	1.95%	7



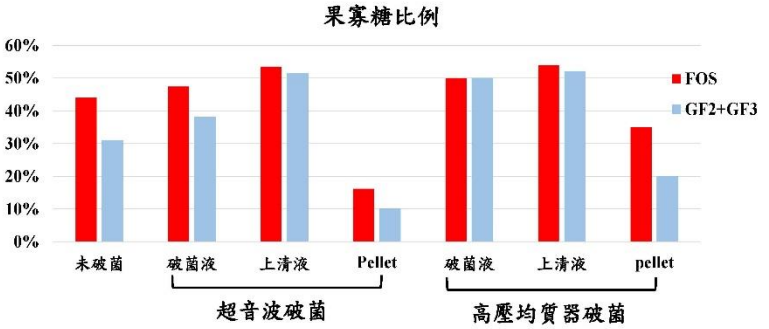
圖一. 菌體破壁果寡糖轉換結果。經破菌後FOS有提升，且破菌離心上清液FOS占總糖53.5%，破菌離心pellet的轉換效率則是有降低的情形。

表三、硫銨沉澱法酵素活性

樣品		酵素活性 (IU)	可溶性蛋白 (mg/g菌體)	回收率	酵素比活性 (IU/mg 蛋白)
冷凍菌塊 未破菌		5125	9.99	100%	513
冷凍菌塊 超音波破菌		5125	16.49	100%	310
破菌後離心上清液		3875	2.34	75.6%	1656
硫銨沉澱	20%	61	0.09	1.20%	6450
	30%	36	0.1	0.70%	3630
	40%	48.5	0.22	0.90%	2170
	50%	46	0.26	0.90%	1750
	60%	14.5	0.14	0.30%	1020



圖二、硫銨沉澱法果寡糖轉換效果。上清液加入30%、40%、50%、60%硫酸銨沉澱後酵素轉換果寡糖效率提高，FOS皆占總糖超過50%，而加入20%硫酸銨沉澱酵素組別則是呈現轉換效率降低的情形。



圖三、工業化大量破菌系統測試。使用高壓均質器進行20 L酵素液破菌，高壓均質器破菌液FOS轉換占總糖50%，相較於超音波破菌液有提高效率，且上清液組別FOS轉換維持54%，而Pellet組別則是FOS轉換從16%提高至35%，因此使用高壓均質器進行大量破菌不會降低FOS轉換，可用至工業化流程中。

結論

本次研究實驗室保存菌株*Aspergillus japonicas*，經破菌處理後，有提高蔗糖轉換的效率。未來可繼續探討酵素的轉換適合條件。將會在未來進行其他條件對酵素的影響，以及工業上的製成工序。

Lutein及SFN在視網膜上皮細胞對抗藍光及光毒素 All-trans retinal的保護機轉與細胞氧化還原態的關係

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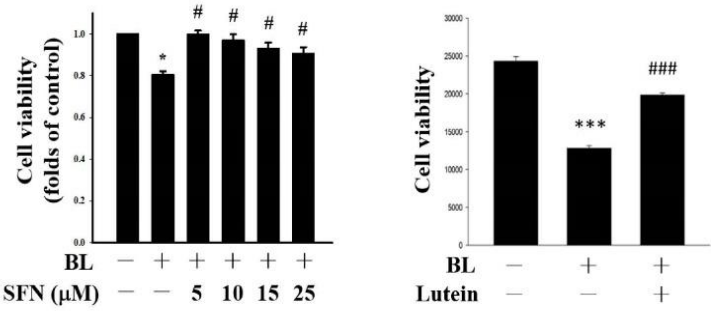
摘要

老年黃斑部病變（Age-related macular degeneration, AMD）是造成已開發國家中老年人視力退化甚至失明的主因。隨著科技的進步，現代人經常長時間接觸手機、電腦、電視等3C產品，而3C產品中的藍光波長短，能量高，越來越多文獻指出過度暴露於藍光下會對視網膜造成氧化壓力，甚至引發細胞凋亡。此外，眼睛在受強光照射後，會產生活性大的副產物all-trans retinal（atRAL），累積於視網膜色素上皮細胞中，誘導氧化壓力及脂質的過氧化（lipid peroxidation），而導致視網膜的損傷。

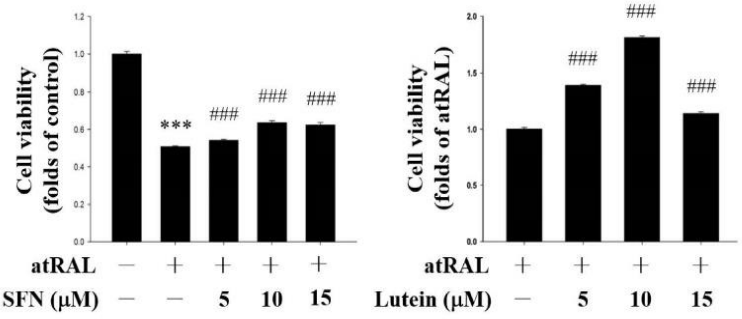
蘿蔔硫素（Sulforaphane, SFN）具有抗氧化的作用，可藉由活化Nrf2-ARE pathway來提升細胞抗氧化能力，而減少細胞內氧化壓力而使細胞免於凋亡。葉黃素（Lutein）高濃度存在於黃斑部中，眾多文獻指出lutein在細胞中可作為藍光過濾器與抗氧化劑來保護視網膜細胞。

本篇目的在於探討SFN與lutein是否可改變RPE細胞的抗氧化能力，保護細胞免於藍光或atRAL引起的氧化傷害。

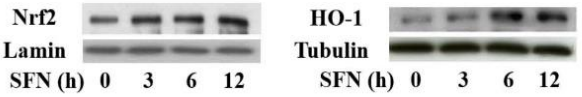
實驗結果



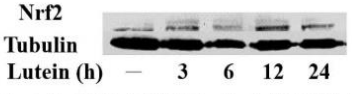
圖一、SFN及Lutein可減少藍光所造成的細胞死亡



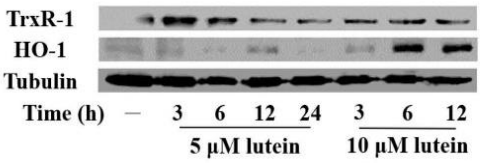
圖二、SFN及Lutein可減少atRAL所造成的細胞死亡



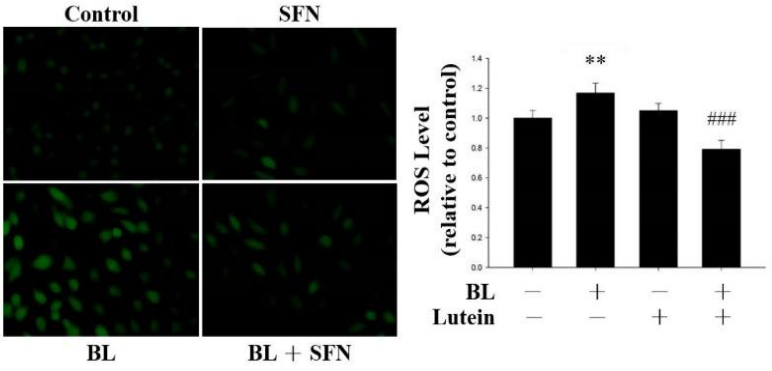
圖三、SFN可增加RPE細胞內抗氧化蛋白及核內Nrf2表現



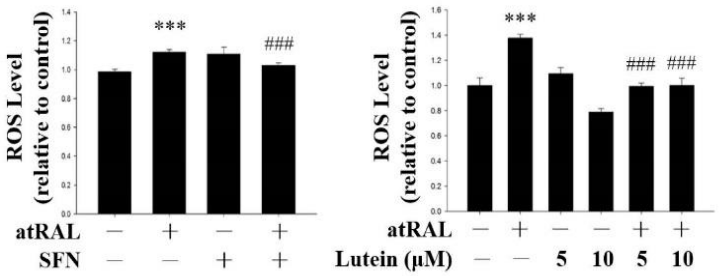
圖四、Lutein可增加RPE細胞內Nrf2蛋白表現



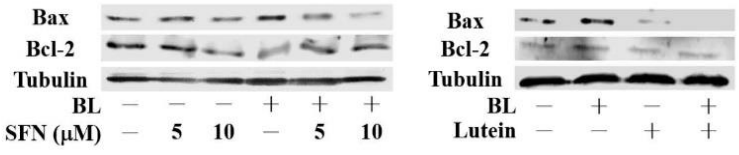
圖五、 Lutein可增加RPE細胞內抗氧化蛋白表現

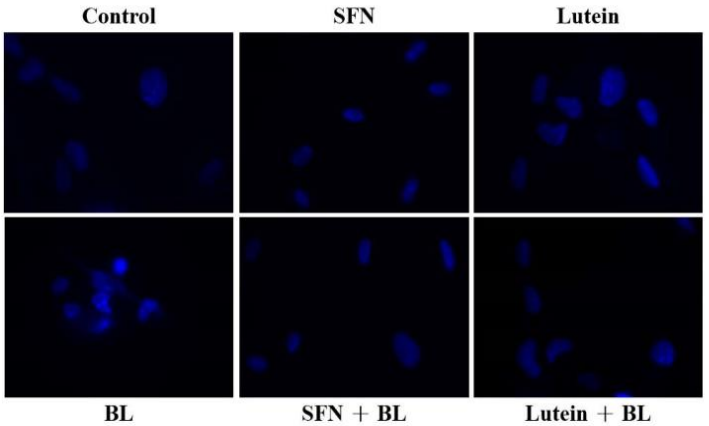


圖六、SFN及Lutein可減少藍光所造成的ROS



圖七、SFN及Lutein可減少atRAL所造成的ROS





圖八、SFN及Lutein可降低藍光所引起的細胞凋亡

結論

藍光會對ARPE-19細胞造成氧化壓力使細胞凋亡，atRAL亦會造成ARPE-19細胞的氧化傷害，而SFN及lutein可以增加細胞的抗氧化能力，以保護細胞免於死亡。

Evaluation of the activity of baicalein and liophilic baicalein derivatives together with anti-cancer drug 5-FU in colorectal cancer cell

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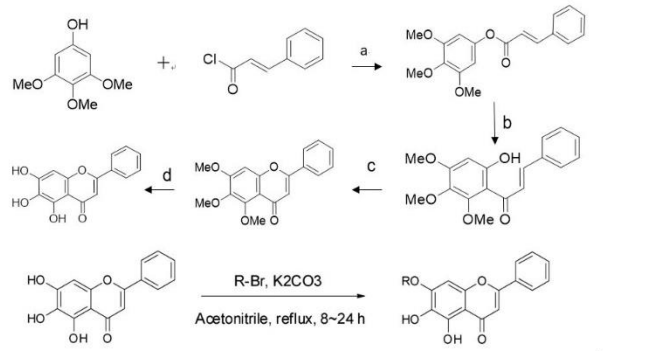
INTRODUCTION

Colorectal cancer is the third most common type of adult cancer in Taiwan. Conventional ways to treat colorectal cancer are surgery, radiation, chemotherapy, targeted therapy, but deleterious toxicity of long term chemotherapeutics compels researchers to develop more efficient drugs with minimized toxicity. For example, 5-FU what is an existing effective clinical drug, but it was previous report detected induction of free radicals and oxidative stress after 5-FU treatment of primary human cardiac cells. Thus, in order to improve this problem, we use natural active substances baicalein, which has been widely used as a traditional Chinese medicine for inflammation, infectious diseases and anti-oxidation. Furthermore, Baicalein have strong anti-tumor effects in cancers, including in breast cancer, prostate cancer, pancreatic cancer, esophageal squamous cell carcinoma and burkitt lymphoma. In this study we modified baicalein with o-alkyl group or terpenyl group, which were screened for an anti-oxidation test was carried out, and it was found that the anti-oxidation ability would not be removed. At the same time, it were screened for cytotoxicity in *vitro* on human cancer cell line. The cytotoxicity of baicalein derivatives were assayed against human colon cancer cell line (DLD-1) by MTT assay. Among them, compound 2c and 3b was the most active compound on cytotoxic activity of human colon cancer cell line. After combine with 5-FU, low dose of 2c and 3b significantly increases 5-FU cytotoxicity and the synergy treatment index CI is also less than 1, especially 2c can reach 0.4 at relatively high doses, which has obvious synergistic effects. In conclusion, the results indicated 2c and 3b could effectively increases 5-FU cytotoxicity in human colon cancer cells and may be a potent anticancer agent.

MATERIALS AND METHODS

Scheme 1: Synthesis of 2-/3-substituted juglone derivatives.



(a) Toluene, reflux, 1h.
(b)10%BF_3·OEt_2, toluene, reflux, 3h.
(c) I_2, cat., DMSO, reflux, 3h.
(d)47%HBr, CH_3COOH, TBAI, reflux, 7h.

Bai-C_5	R= Isoprenyl
Bai-C_{10}	R= geranyl
Bai-C_{15}	R= farnesyl
Bai-C_{10}'	R= decyl
Bai-C_{12}	R=dodecyl

RESULTS

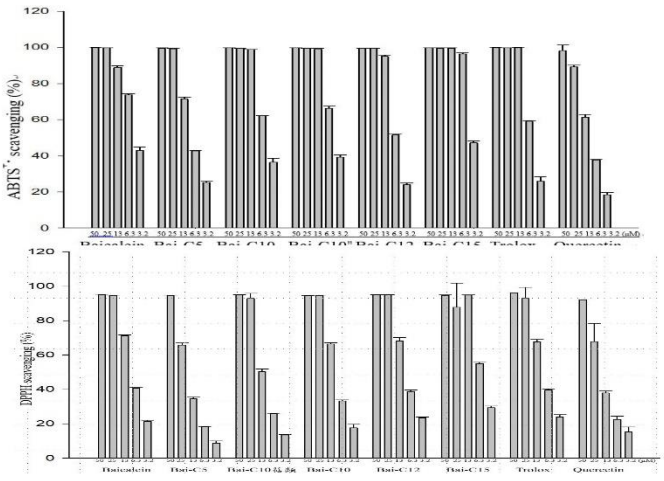


Figure 1.. Anti-oxide effect of baicalein and its derivatives in $ABTS^{+}$ and DPPH

Table 2 Physical properties of baicalein and its derivatives.

	R	MW	cLog*P*[a]	MP (°C)
Baicalein (1)	H	270.24	3.00	270.2~272.4
Baicalein-C_5 (2a)	Isoprenyl	338.34	3.40	155.9~157.3
Baicalein-C_{10} (萜類)(2b)	Geranyl	406.47	4.93	133.6~134.9
Baicalein-C_{15} (2c)	Farnesyl	474.59	6.45	105.9~106.9
Baicalein-C_{10} (3a)	Decyl	410.50	5.91	116.8~118.2
Baicalein-C_{12} (3b)	Dodecyl	438.56	6.75	119.6~120.8

Table 3 Anti-oxide effect of baicalein and its derivatives.

Compound	IC_{50}(μM) DPPH	ABTS+
Baicalein (1)	7.88 ± 0.46	4.24 ± 0.25
Baicalein-C_5 (2a)	19.37 ± 0.88	7.79 ± 0.106
Baicalein-C_{10} (2b)	12.53 ± 0.11	6.21 ±1.10
Baicalein-C_{15} (2c)	13.09 ± 2.14	5.49 ±0.06
Baicalein-C_{10} (3a)	10.66 ± 1.69	6.00 ±1.30
Baicalein-C_{12} (3b)	10.13 ± 2.04	7.33 ± 1.28
Trolox	10.85 ± 2.10	4.99 ± 1.53
Quercetin	19.91 ± 2.23	10.46 ± 1.9

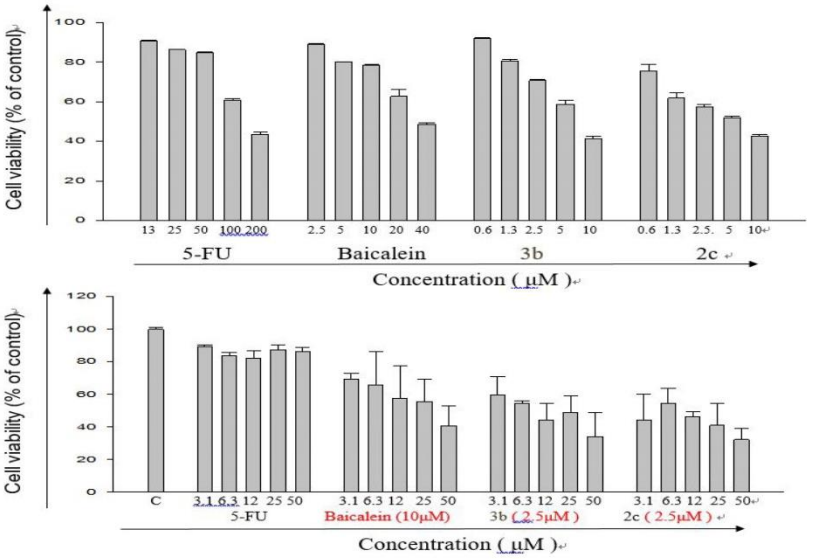


Figure 2. Cytotoxicity of DLD-1 cells treated with plumbagin and **2c** and **3b** and 5-FU combine with **2c** and **3b** for 48 and 72 h.

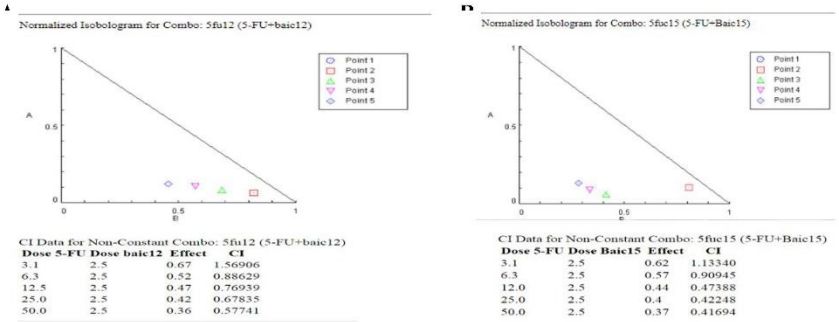


Figure 3. CI of 5-FU combine with baicalein derivatives in DLD-1

CONCLUSIONS

In this study, we synthesized a series of 7-O- alkyl group or terpenyl group. Among the compound 2c and 3b have better cytotoxicity and same antioxidant ability of baicalein. Furthermore, according to CI value exhibit combined treatment with 5-FU significantly increased the cytotoxicity of 5-FU on colon cancer DLD-1. The present results indicated the compound 2c and 3b is a novel anticancer combined drugs of candidate for colon cancer in the future.

Co-treatment of vorinostat with doxorubicin synergistically enhances anticancer effect in bladder cancer cells.

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Abstract

Bladder cancer is the 10th highest incidence of cancer worldwide. Approximately 70% of bladder cancers are diagnosed as non-muscle invasion which are treated by transurethral resection followed by intravesical therapy. Doxorubicin (DOXO) is one of the effective cytotoxic drugs used in intravesical therapy, but the cardiotoxicity and nephrotoxicity limit its therapeutic dosages. Vorinostat (SAHA) is an oral anti-cancer drug for cutaneous T-cell lymphoma. Though SAHA is not applied for bladder cancer, its pharmacological mechanism is different from DOXO. So, this study would like to investigate the combination effect of DOXO and SAHA in human bladder cancer cells, and discuss their mechanisms. In cell viability assay, the alive cell of BFTC 905 cells and 5637 cells were dose-dependently decreased by DOXO and SAHA individually. In the IC20 of each drug, DOXO plus SAHA synergistically induced cell death comparing to the single drug treatment in BFTC 905 and 5637 cells. In Western blot analysis, the combined treatment of DOXO and SAHA also synergistically enhanced the apoptosis-related proteins expression, included cleaved-PARP, cleaved-caspase 3 and γ-H2AX. After a pan caspase inhibitor (Z-VAD-FMK) pre-treatment, the expression of apoptosis-related proteins significantly downregulation, meanwhile, the cell death was partially reversed. On the contrary, the pre-treatment of an anti-oxidant (N-acetylcysteine, NAC) minimally improved cell viability on SAHA treatment and combined drugs treatment in BFTC 905 cells, but there was no effect on 5637 cells. Taken together, these results indicated that the synergistic effect of DOXO and SAHA on inducing cell death maybe majorly through caspase 3-PARP pathway but not ROS. Our results showed that the combination of DOXO with SAHA might represent a novel regimen for the treatment of urothelial carcinoma, and the effect will be analyzed in orthotopic and/or xenograft mice models.

Introduction

❑ **Mechanism of Doxorubicin (DOXO)**
Doxorubicin intercalated of its planar rings in the minor groove of the DNA double helix, which can stabilizes the topoisomerase II-DNA complex, inhibiting the progression of the S-phase of the cell cycle. The topoisomerase II-doxorubicin-DNA complex can induce DNA double strand breaks. 2016. *Drug Resist Updat* **29**, 90-106

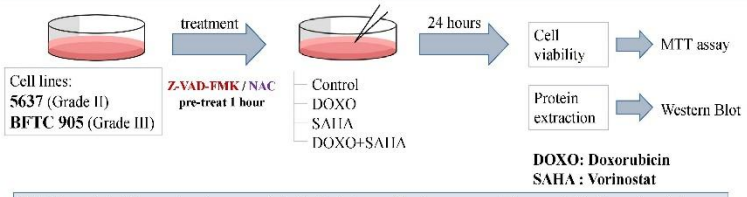
❑ **Dose-limiting side effect of Doxorubicin**
The most common side effects are acute nausea and vomiting, neurologic disturbances, cumulative cardiotoxicity, bone marrow aplasia, leukopenia, neutropenia, thrombocytopenia and anemia. Pharmacokinetics of doxorubicin is characterized by a very short half-life in circulation with extensive, non-selective tissue distribution. Therefore, effective therapy with doxorubicin will often require high doses, which can further aggravate adverse toxic side effects due to the lack of selectivity of this drug. 2009. *Curr Med Chem* **16**, 3267-3285
2016. *Drug Resist Updat* **29**, 90-106

❑ **HDAC inhibitor and its effects**
Inhibition of histone deacetylase enzyme causes chromatin decondensation, which regulated the transcription of genes involved in cell survival and apoptosis. HDAC inhibitors (eg. SAHA) seem to be promising anti-cancer drugs particularly in the combination with other anti-cancer drugs. 2017. *Int J Mol Sci* **18**(7), 1414
2003. *Cancer Res* **63**(21), 7291-7300

Aim

To investigate whether the combination of DOXO and SAHA have a synergistic effect in human bladder cancer cells, and discuss their mechanism.

Materials and Methods



Statistic analysis: The experiments were conducted in triplicates, and the data are presented as mean ± SE. Comparisons between groups were performed by Student's two-tailed t test. ($^{a, aa, aaa}$Significant at $P < 0.05$, 0.01, or 0.001 versus control, respectively; $^{b, bb, bbb}$Significant at $P < 0.05$, 0.01, or 0.001 versus Z-VAD-FMK treatment group, respectively; $^{c, cc, ccc}$Significant at $P < 0.05$, 0.01, or 0.001 versus NAC treatment group, respectively; $^{*, **, ***}$Significant at $P < 0.05$, 0.01, or 0.001, respectively; n=3)

Results

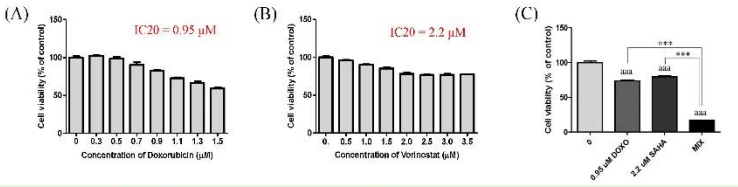


Figure 1. Cell viability analysis of DOXO and SAHA in BFTC 905 cells.
(A) Effect of DOXO in cell viability was determined by using MTT assay. Cell lines were treated with different doses of DOXO (from 0 to 1.5 μM) and incubated for 24 hours. (B) Through MTT assay, effect of SAHA was determined. Cell lines were treated with different doses of SAHA (from 0 to 3 μM) and incubated for 24 hours. We selected a concentration of DOXO and SAHA with 80% viability for follow-up combination experiments. (C) BFTC 905 cells were treated with 0.95 μM DOXO and/or 2.2 μM SAHA for 24 hours, and the cell viability was assessed using a MTT assay. Bars represent the mean ± SE of 3 experiments.

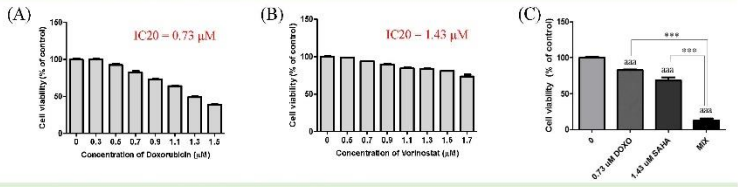


Figure 2. Cell viability analysis of DOXO and SAHA in 5637 cells.
(A) Effect of DOXO in cell viability was determined by using MTT assay. Cell lines were treated with different doses of DOXO (from 0 to 1.5 μM) and incubated for 24 hours. (B) Through MTT assay, effect of SAHA was determined. Cell lines were treated with different doses of SAHA (from 0 to 1.7 μM) and incubated for 24 hours. We selected a concentration of DOXO and SAHA with 80% viability for follow-up combination experiments. (C) 5637 cells were treated with 0.73 μM DOXO and/or 1.43 μM SAHA for 24 hours, and the cell viability was assessed using a MTT assay. Bars represent the mean ± SE of 3 experiments.

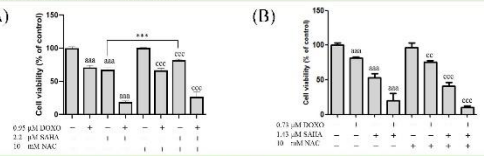


Figure 3. . DOXO combined with SAHA on inducing cell death were not mainly through ROS induced.
(A) The cell viability was determined by using MTT assay. BFTC 905 cells were pretreated with 10 mM NAC then incubated with 0.95 μM DOXO and/or 2.2 μM SAHA for 24 hours. Bars represent the mean ± SE of 3 experiments.
(B) The cell viability was determined by using MTT assay. 5637 cells were pretreated with 10 mM NAC and treated with 0.73 μM DOXO and/or 1.43 μM SAHA for 24 hours. Bars represent the mean ± SE of 3 experiments.

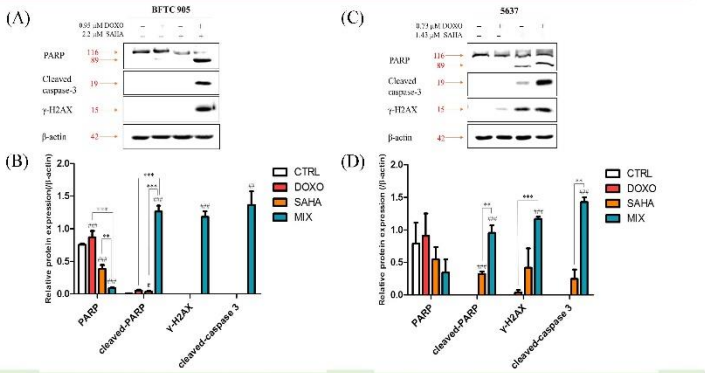


Figure 4. DOXO combined with SAHA upregulated the expression of cleaved poly (ADP-ribose) polymerase (PARP), γ-H2AX and cleaved-caspase 3 in human bladder cancer cells.
(A) Through Western blot, analyzing 0.95 μM DOXO and/or 2.2 μM SAHA treated BFTC 905 cells for 24 hours on the protein expression of PARP, γ-H2AX and cleaved-caspase 3. (B) Co-treatment of DOXO and SAHA can significantly induced cleaved PARP, γ-H2AX and cleaved-caspase 3 protein expression. (C) Through Western blot, analyzing 0.73 μM DOXO and/or 1.43 μM SAHA treated 5637 cells for 24 hours on the protein expression of PARP, γ-H2AX and cleaved-caspase 3. (D) Co-treatment of DOXO and SAHA can significantly induced cleaved PARP, γ-H2AX and cleaved-caspase 3 protein expression.

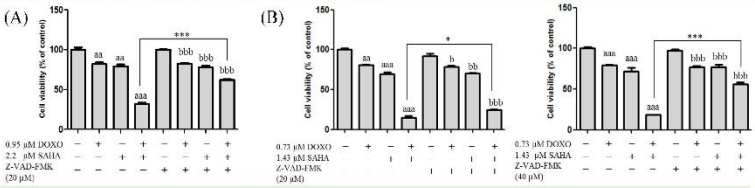


Figure 5. Z-VAD-FMK partly restored the cell viability of combined treatment in BFTC 905 cells and 5637 cells.
(A) The cell viability was determined by using MTT assay. BFTC 905 cells were pretreated with 20 μM Z-VAD-FMK for 1 hour then incubated with 0.95 μM DOXO and/or 2.2 μM SAHA for 24 hours. Bars represent the mean ± SE of 3 experiments.
(B) The cell viability was determined by using MTT assay. 5637 cells were pretreated with 20 μM or 40 μM Z-VAD-FMK for 1 hour and treated with 0.73 μM DOXO and/or 1.43 μM SAHA for 24 hours. Bars represent the mean ± SE of 3 experiments.

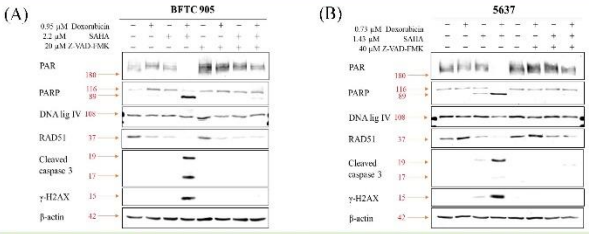


Figure 6. The expression of PAR, cleaved poly (ADP-ribose) polymerase (PARP), RAD51 and γ-H2AX in combined treatment of human bladder cancer cells were depended on caspase 3.
(A) After pretreatment with 20 μM Z-VAD-FMK, analyzing 0.95 μM DOXO and/or 2.2 μM SAHA treated BFTC 905 cells for 24 hours on the protein expression through western blot. 20 μM Z-VAD-FMK almost entirely reduced the protein expression of cleaved PARP and γ-H2AX; protein expression of PAR and RAD51 also partially reversed in combined treatment of BFTC 905 cells. (B) After pretreatment with 40 μM Z-VAD-FMK, analyzing 0.73 μM DOXO and/or 1.43 μM SAHA treated 5637 cells for 24 hours on the protein expression through western blot. 40 μM Z-VAD-FMK almost entirely reduced the protein expression of cleaved PARP and γ-H2AX; protein expression of PAR and RAD51 also partially reversed in combined treatment of 5637 cells.

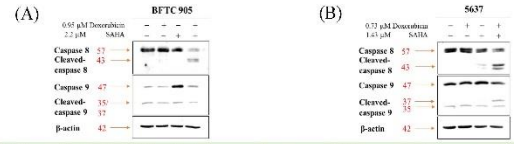


Figure 7. DOXO combined with SAHA activated caspase 8 or caspase 9 in human bladder cancer cells.
(A) 0.95 μM DOXO and/or 2.2 μM SAHA treated BFTC 905 cells for 24 hours on the protein cleavage of caspase 8 and caspase 9. Co-treatment of DOXO and SAHA can significantly induced caspase 8 protein cleavage. (B) 0.73 μM DOXO and/or 1.43 μM SAHA treated 5637 cells for 24 hours on the protein cleavage of caspase 8 and caspase 9. Co-treatment of DOXO and SAHA can significantly induced caspase 8 and caspase 9 protein cleavages.

Conclusion

- ❑ Combination of Doxorubicin and Vorinostat is effective in reducing cell viability of BFTC 905 and 5637 cells.
- ❑ Doxorubicin combined with Vorinostat significantly up-regulated protein expression of cleaved poly (ADP-ribose) polymerase (PARP), γ-H2AX and cleaved caspase 3.
- ❑ Z-VAD-FMK partially reversed the cell viability and completely reduced the protein expression of cleaved poly (ADP-ribose) polymerase (PARP) and γ-H2AX; protein expression of PAR and RAD51 also partly reversed in combined treatment of Doxorubicin and Vorinostat.
- ❑ Combination of Doxorubicin and Vorinostat induced apoptosis through caspase 8/9 activation.
- ❑ Doxorubicin combined with Vorinostat in reducing cell viability of BFTC 905 and 5637 cells were mainly through caspase 3-PARP pathway but not ROS.
- ❑ Combined effect of Doxorubicin and Vorinostat on inducing cell death were majorly through activated caspase 8/9-caspase 3-PARP-γ-H2AX pathway.

Sub-chronic toxicity and immunological successful-aging assessments of Shell Ginger (*Alpinia zerumbet*)

月桃亞慢性毒性及在成功老化上之免疫功能評估

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摘要

日本人民的平均壽命在世界中是數一數二的長壽，其中又以沖繩的居民為最長壽，而造成這樣的原因，主要可能和沖繩居民的飲食有密切的關連。在沖繩，有特殊的”沖繩飲食”，被認為是影響沖繩居民長壽的關鍵，其中又以月桃(*Alpinia zerumbet*)為一特有的食材，因此近年來興起許多以月桃為題材的相關研究。過去有許多對於月桃之抗氧化及抗癌研究，但對於免疫評估相關之完整研究，以及其免疫功能對於延緩老化之關聯探討尚缺乏，又因對於月桃葉食用安全有疑慮，因此本研究進行月桃葉90天餵食毒性試驗。試驗中，每日給予飲食最高濃度5%月桃葉之小鼠，其採食量均無異常，期間體重平均，外觀無顯著變化。在臨床病理檢測中也無病理變化，顯示長期食用月桃葉並不會對身體造成危害。月桃葉之95%乙醇萃取物進行細胞毒性試驗，所有測試之細胞株細胞毒性IC50濃度幾乎都高於500 μg/ml，在月桃葉萃取物毒性範圍內，小鼠巨噬細胞RAW264.7可顯著抑制LPS刺激之NO產生，並提升吞噬能力。並且在動物實驗中也顯示出月桃葉處理組能減少細胞媒介免疫反應造成的腫脹，提升NK cell的活性，並且增加調節型T細胞(Treg)數量。實驗結果顯示，不論是減輕發炎反應或是增加抗癌效果，皆有幫助成功老化的趨勢，並且長期食用不會對身體造成危害。

前言

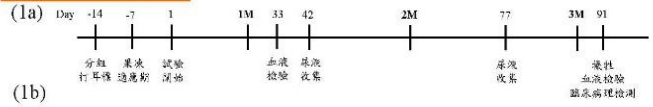
月桃(*Alpinia zerumbet*)中的植物化學物質可分為兩大類，卡瓦酸內酯類(Kavalactones)及非卡瓦酸內酯類(Non-kavalactones)。卡瓦酸內酯類(Kavalactones)含有兩個主要成分，Dihydro-5,6-dehydrokawain (DDK)及其衍生物5,6-Dehydrokawain (DK)，含量最高，且在月桃葉中含有豐富的DDK及DK。過去研究(Hsu, et al. 1994)指出DDK和DK有神經性毒性，甚至影響小鼠行為表現，因此本研究針對月桃葉進一步進行毒性評估。文獻(Nguyen, et al. 2014)指出DK經過胃酸及肝臟代謝後會轉化成Hispidin，推測可能是月桃葉進到體內作用時一個重要的成分。在老年人身上容易發生的健康問題大多與體內慢性發炎有關，近年來延緩老化之研究著重於提升老年人的健康品質，特別是減輕體內慢性發炎的問題，研究指出Hispidin有抑制發炎及免疫調控的效果，因此本研究目的為探討月桃葉中DDK及其衍生物DK及Hispidin等成分對於延緩老化之免疫調控效果。

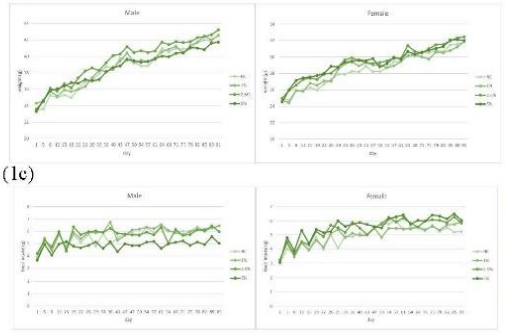
材料與方法

月桃葉乾燥後打磨成細粉，製成不同濃度月桃葉果凍，餵食ICR小鼠90天進行餵食毒性試驗。期間觀察生理狀況，並於犧牲後進行臨床病理檢驗。95%乙醇萃取月桃葉，利用HPLC分析成分，並利用95%乙醇回溶萃取物進行體外試驗，使用五種細胞株進行MTT assay測細胞毒性，以及RAW264.7小鼠巨噬細胞吞噬作用及NO產生試驗。免疫評估動物試驗則使用BALB/c小鼠餵食月桃葉萃取物，進行先天免疫及後天免疫兩部分評估，先天免疫評估試驗包含自然殺手細胞活性試驗、吞噬細胞吞噬能力試驗及細胞媒介免疫反應(Delayed-type hypersensitivity(DTH)足腫試驗)；後天免疫評估試驗包含抗體分泌試驗、T細胞次族群分析及淋巴細胞增生試驗，並且測定免疫調節之重要細胞激素基因表達。

結果與討論

亞慢性毒性試驗



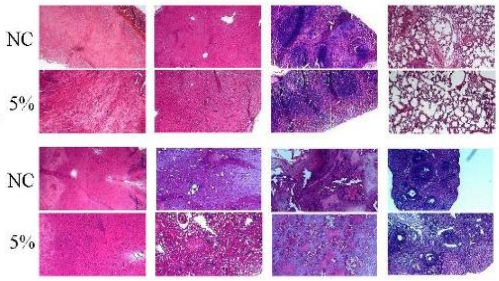


圖一、(1a)90天餵食毒性試驗，每日觀察動物狀態，期間進行兩次抽血檢驗及尿液收集，並於動物犧牲後進行臨床病理檢測；(1b)試驗期間每週為小鼠秤重兩次，各組體重平均；(1c)飼糧攝取量也無顯著差異。

表一、小鼠每日攝取月桃葉重量

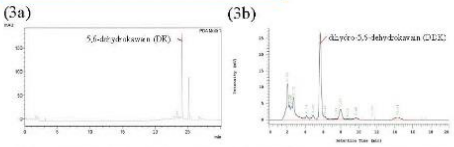
月桃葉每日攝取量(每隻小鼠)	Male	Female
1%	0.065-0.13 g	0.13-0.2 g
2.5%	0.163-0.325 g	0.325-0.5 g
5%	0.35-0.5 g	0.35-1 g
最高攝取: 31.25 g/kg BW		

小鼠在90天月桃葉餵食過程中，體重(圖1b)及飼糧攝取(圖1c)皆正常，計算月桃葉攝取量每日最高可達31.25 g/kg BW(表一)。每天觀察小鼠外觀無異常，排泄正常，尿液檢測結果(未顯示)也正常。血液檢測結果在第一個月，餵食月桃葉組別的白血球數量有變化，但在第三個月檢測時，即恢復正常，並且血清生化檢驗各項皆正常(未顯示)。最後，餵食月桃葉對各個器官並不會造成損傷(圖二)，由以上結果可得結論，在安全劑量內長期食用月桃葉並不會對身體造成慢性毒性。



圖二、不同器官組織切片染色NC組及5%組比較(1%及2.5%皆與NC組無差異)，上排由左至右分別為: 心、肝、脾、肺；下排由左至右為: 腦、腎、睾丸(公鼠)、卵巢(母鼠)，NC組和月桃葉處理組器官皆正常。

月桃葉95%乙醇萃取物分析



圖三、HPLC檢測kavalactones成分：(3a)5,6-Dehydrokawain (DK)；(3b) Dihydro-5,6-dehydrokavain (DDK)。

表二、月桃葉95%乙醇萃取物中kavalactones含量

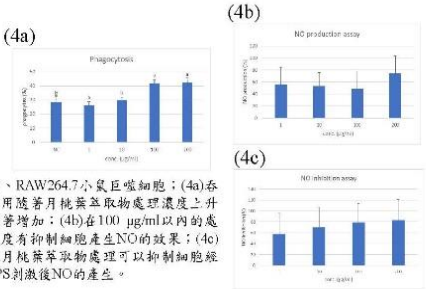
kavalactones在月桃葉萃取物中含量(%)	
dihydro-5,6-dehydrokavain (DDK)	3.87%
5,6-dehydrokawain (DK)	0.31%

月桃葉委託金屬工業研究發展中心(嘉義產業創新研發中心)天然物創新應用研究所協助萃取及測定DK含量(圖3a)，DDK則是經過分離純化後，測NMR鑑定結構(未顯示)，再利用HPLC定量計算DDK在月桃葉萃取物中含量(表二)，可見DDK與DK在萃取物中含量相差將近10倍。

體外細胞實驗

表三、細胞毒性試驗(MTT assay)

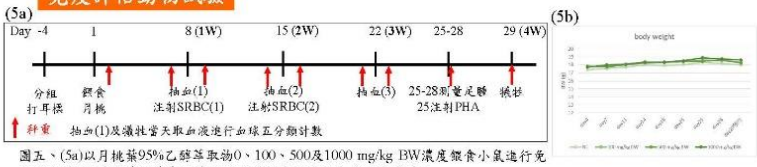
IC50 (μg/ml)		95%乙醇萃取物
MTT		
淋巴系免疫細胞	YAC-1	375.5
纖維母細胞	RAW264.7	600.5
體細胞	肝細胞 BNL CL.2	921.8
體細胞	脂肪前驅細胞 3T3-L1	1107
體細胞	乳癌細胞 JC	645.4



圖四、RAW264.7小鼠巨噬細胞：(4a)吞噬作用隨著月桃葉萃取物處理濃度上升而顯著增加；(4b)在100 μg/ml以內的處理濃度有抑制細胞產生NO的效果；(4c)並且月桃葉萃取物處理可以抑制細胞經過LPS刺激後NO的產生。

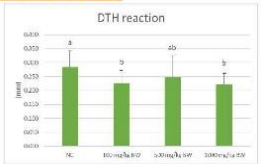
細胞毒性試驗使用五種不同的細胞株，其中兩株免疫細胞的毒性IC50濃度較低，可見月桃葉萃取物對免疫細胞影響較大，體細胞中，萃取物對肝細胞BNL CL.2及脂肪前趨細胞3T3-L1的毒性較低，但對乳癌細胞的毒性卻較強，這證明了月桃葉萃取物能抑制癌細胞的生長，與許多先前抗癌的研究相符合。另外，萃取物能增加RAW264.7小鼠巨噬細胞的吞噬能力並抑制NO的產生。

免疫評估動物試驗



圖五、(5a)以月桃葉95%乙醇萃取物0、100、500及1000 mg/kg BW濃度餵食小鼠進行免疫評估試驗；(5b)實驗中每週為小鼠秤重兩次，各組小鼠體重平均。

先天免疫評估



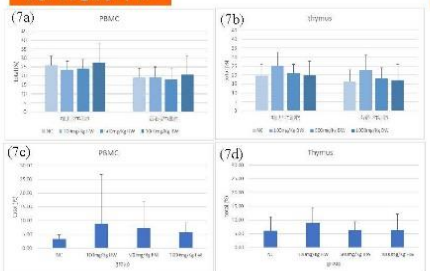
圖六、利用PHA趨化T cell聚集並產生IFN-γ、TNF-α等發炎細胞激素，反應後期造成吸引巨噬細胞浸潤組織，造成腫脹。

表四、自然殺手細胞活性

NK cell activity			
E/T ratio	1	5	10
NC	35.08±30.73	31.33±31.01	33.51±24.47
100mg/Kg BW	37.21±31.08	35.05±32.34	36.50±28.22
500mg/Kg BW	37.76±29.44	32.95±30.52	30.51±22.55
1000mg/Kg BW	37.41±31.91	35.89±32.14	34.45±26.07

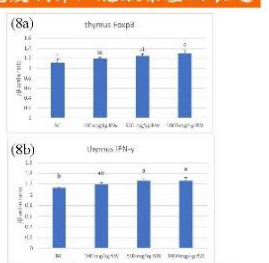
月桃葉萃取物餵食之組別小鼠吞噬細胞吞噬能力並無差異(未顯示)，但DTH足腫之腫脹程度都有顯著下降(圖六)，說明萃取物有抑制發炎反應的效果；並且提升自然殺手細胞活性，文獻指出(Nguyen et al. 2014)，DK經過胃酸水解，再被肝臟代謝以後，會成為hispidin，又另一文獻指出(Gründemann et al. 2016)，hispidin可以提升NK cell活性，說明hispidin可能在體內作為一調節因子。

後天免疫評估



圖七、(7a,b)PBMC及胸線T細胞次族群分析(輔助型：毒殺型T細胞)；(7c,d) PBMC及胸線T細胞次族群分析(調節型T細胞)

免疫調節細胞激素基因表達



圖八、胸腺細胞(8a)Foxp3基因表現量；(8b) IFN-γ基因表現量

後天免疫評估中，小鼠餵食月桃葉萃取物對淋巴細胞增生(未顯示)及T細胞次族群比例(7a,b)無影響，但增加調節型T細胞(regulatory T cell (Treg))比例(7c,d)，並且上調IFN-γ基因表現量(8a)及Treg之重要調節蛋白Foxp3基因表現(8b)，可控制過度的發炎反應。

結論

月桃葉在安全劑量內長期食用並不會對身體造成危害，有抗發炎、提升自然殺手細胞活性(抗癌)等效果，並且能提升調節型T細胞的比例，解決老年人體內慢性發炎的問題，避免慢性疾病的發生，是有潛力發展延緩老化產品的材料，進而達到延年益壽的效果。

資料來源: B. C. Nguyen, N. Taira, S. Tawata, Several herbal compounds in Okinawa plants directly inhibit the oncogenic/aging kinase PAK1. *Drug Discov Ther* **8**, 238-244 (2014). ; C. Grtndemann *et al.*, Effects of Inonotus hispidus Extracts and Compounds on Human Immunocompetent Cells. *Planta Med* **82**, 1359-1367 (2016). ; S. Y. Hsu, M. H. Lin, L. C. Lin, C. J. Chou, Toxicologic studies of dihydro-5,6-dehydrokawain and 5,6-dehydrokawain. *Planta Med* **60**, 88-90 (1994).

The Potential RNA Expression Regulation of Nicotine-Promoted Migration in T24 Bladder Cancer Cells

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Background and Aim

Bladder cancer is a high recurrent cancer with the opportunity to progress to a high metastatic state which will cause higher mortality. Nicotine is the main component of cigarettes and smoking is the most inducing factor for bladder cancer. In the present study, we want to identify whether nicotine promotes bladder cancer cells proliferation and migration, and if any miRNA involves in the nicotine-induced disease progression mechanism.

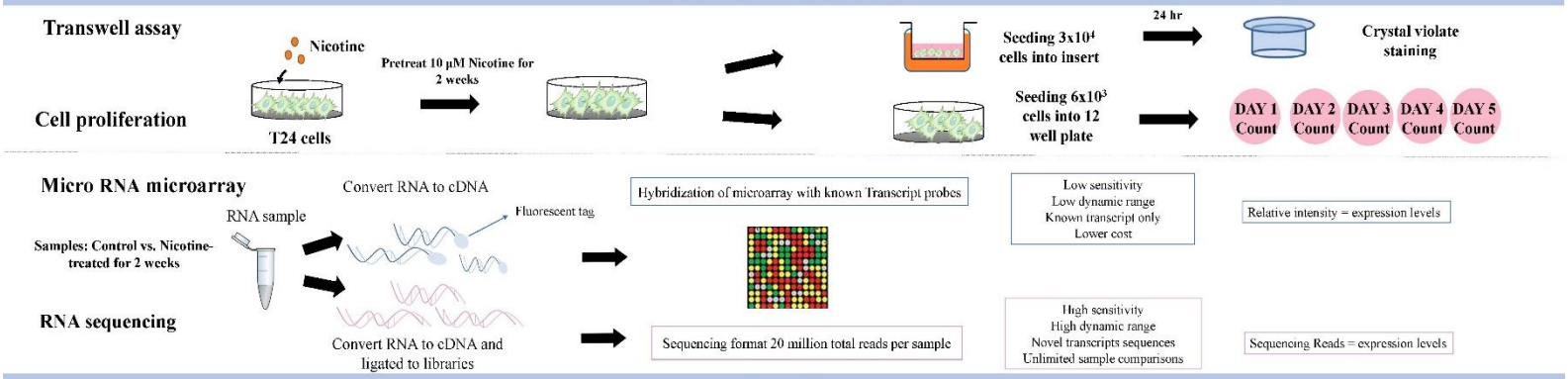
Materials and methods

After pre-treatment of 10 μM nicotine for 2 weeks in T24 bladder cancer cells, performed cell proliferation and metastasis analysis. Three mesenchymal-related protein expression were analyzed by Western blot. Their mRNAs were quantified by quantitative real-time polymerase chain reaction (qPCR). RNAs expression were assayed by RNA sequencing (RNA-Seq) and microRNAs were comprehensive analyzed by array chip.

Results and Conclusion

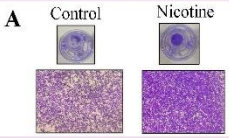
The results showed that T24 bladder cancer cells with 10 μM nicotine treatment for two weeks could promote cell proliferation and cell migration. In Western blot analysis, nicotine could increase the mesenchymal-related protein expression, including N-cadherin, Snail and Vimentin. The qPCR verified that the mRNA expression of these three genes also increased. In the microRNA array chip analysis, the data showed there were 50 up-regulated and 1 down-regulated. In 50 up-regulated genes, after screening by nicotine-increased ratio, normalized intensity, and metastasis-related genes, 9 genes were selected for individual qPCR verification. The only one down-regulated gene was also examined by qPCR. Finally, we got 3 microRNA (miR-21-5p, miR-181a-5p and miR-320d) which were clearly up-regulated by nicotine treatment. In RNA-Seq data, 239 transcripts were down-regulated by nicotine treatment. Intersection of the 239 transcripts and 3 microRNA (miR-21-5p, miR-181a-5p and miR-320d)-interacted RNA, 213 transcripts were selected. Delete the genes with low FPKM values and screen the gene function, we found some candidates (UACA, TFPI, RND3, UFM1) were down-regulated by nicotine. Next, overexpression of miR-320d in T24 cells confirmed that it could increase T24 cell migration, down-regulated the expression of UACA, TFPI, RND3, UFM1 and increased the expression of metastasis-related genes and proteins. Moreover, we added the miR-320d inhibitor to confirm that it can inhibit cell migration and the mRNA expression of metastasis-related genes, but only up-regulated the mRNA expression of RND3 gene. In the future work, the cell migration-related function of T24 bladder cancer cells will be analyzed by using knockdown RND3 expression. The novel findings might provide some new candidates for bladder cancer biomarkers.

Materials and methods



Results

Figure 1. Nicotine promotes T24 cell migration. (A) Transwell assay. Pre-treatment of 10 μM nicotine for 2 weeks in T24 bladder cancer cells, plated 3x10⁴ per transwell for 24 hours. Magnification x40. (B) The quantitative results represent the mean±SD from three independent experiments using the bar graph.**P<0.01.



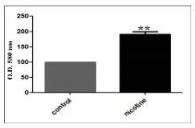


Figure 2. Nicotine promotes T24 cells proliferation. Pre-treatment of 10 μM nicotine for 2 weeks in T24 bladder cancer cells, plated 6x10³/well in 12-well for 5 days. *P<0.05, **P<0.01, ***P<0.001.

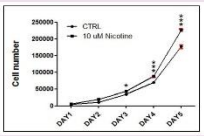


Figure 3. Nicotine promotes N-cadherin, snail, vimentin mRNA expression. Expression levels of N-cadherin, snail, vimentin mRNA by qPCR assay. *P<0.05.

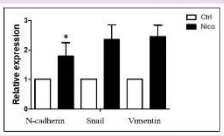
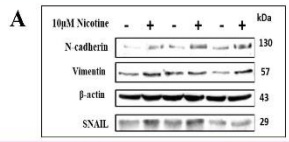


Figure 4. Nicotine promotes N-cadherin, snail, vimentin expression in Western blot analysis. (A) Western blot assay. (B) The quantitative results for western blot.*p<0.05.



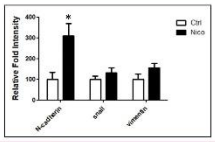


Figure 5. Real-time PCR validation of nicotine-increased miRNA expression from miRNA microarray data. The results indicate that miR-21-5p, miR-181a-5p and miR-320d are up-regulated by nicotine in T24 bladder cancer cells by real-time PCR analysis. *P<0.05.

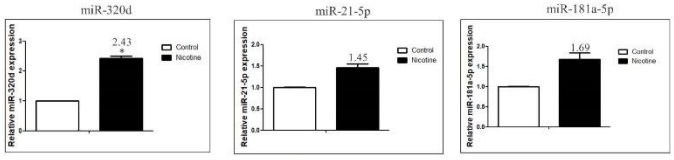


Figure 6. The potential targets of miR-21-5p, miR-320d, miR-181a-5p from RNA sequencing data. Search the potential targets of miR-21-5p, miR-181a-5p, miR-320d from mirRanda, Pita and Targetspy. The potential genes were cross-analyzed with the result of RNA sequencing. Then select the genes related to cell migration.

Fragments Per Kilobase per Million (FPKM)

Gene name	Control	Nicotine	Log_2(N/C)	P-value
TFPI	93.9523	35.9126	-1.387438375	0.00005
HMGA2	206.075	89.5975	-1.201639112	0.00185
RND3	84.0799	37.0314	-1.183011861	0.00005
CST1	274.6	121.078	-1.181394876	0.00005
FGFBP1	99.5443	44.6258	-1.157460676	0.0001
INHBA	107.772	48.6642	-1.14704966	0.00005
MT-RNR2	3815.2	1727.93	-1.142713914	0.00035
UFM1	67.1116	32.3293	-1.053719882	0.00165
UACA	126.204	61.004	-1.048781889	0.0284
LMAN1	111.969	54.7209	-1.032935496	0.00005

TFPI (Tissue Factor Pathway Inhibitor)

RND3 (Rho Family GTPase 3)

UFM1 (Ubiquitin Fold Modifier 1)

UACA (Uveal Autoantigen With Coiled-Coil Domains And Ankyrin Repeats)

Figure 7. Nicotine induced UACA, RND3, UFM1 and TFPI down-regulated using real-time PCR detection. Expression levels of UACA, RND3, UFM1 and TFPI mRNA by qPCR. **P<0.01.

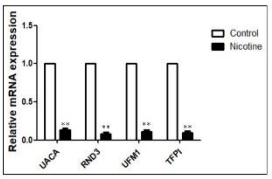


Figure 8. Overexpression miR-320d promoted T24 bladder cancer cell migration. (A) Transwell assay. Pre-treatment of 10 μM nicotine for 2 weeks in T24 bladder cancer cells, plated 3x10⁴ per transwell for 24 hours. Magnification x40. (B) The quantitative results represent the mean±SD from three independent experiments using the bar graph.*P<0.05.

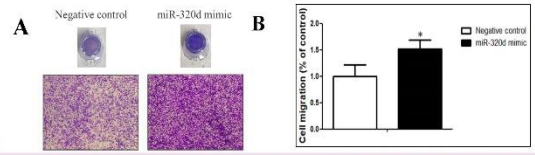


Figure 9. Overexpression miR-320d induced UACA, RND3, UFM1, TFPI down-regulated using real-time PCR detection. Expression levels of UACA, RND3, UFM1 and TFPI mRNA by qPCR. *P<0.05, **P<0.01.

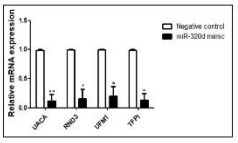
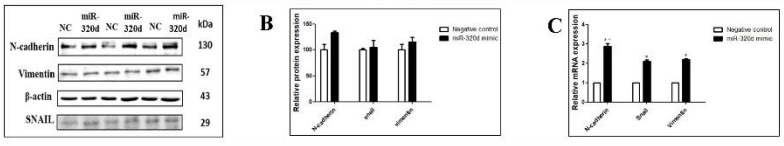


Figure 10. Overexpression miR-320d promotes N-cadherin, snail, vimentin expression in Western blot analysis and real-time PCR detection . (A) Western blot assay. (B) The quantitative results for western blot.*p<0.05. (C) Expression levels of mRNA by qPCR. *P<0.05, **P<0.01.



Conclusion

Figure 11. Schematic showing the potential mechanism of accelerated migration by nicotine. Nicotine increases miR-320d expression which might target RND3 leading to down-regulation. Therefore, the miR-320d-RND mRNA regulation mechanism might play a role in nicotine-promoted migration in T24 bladder cancer cells.