行政院國家科學委員會專題研究計畫成果報告

題目：合成 HP 類緣化合物作為新型細胞分化劑

Synthesis of hemoregulatory peptide analogs as cell differentiation agents

計畫編號：NSC 90-2320-B-039-038

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主持人：龍鳳娣

一、中文摘要

Antineoplastons 為人體及體液中自然產生之化學物質，經證實可選擇性地抑制腫瘤細胞之生長並誘導腫瘤細胞進行終末細胞分化。研究發現 Antineoplastons 抑制腫瘤細胞生長並誘導腫瘤細胞分化之反應機轉涉及 antineoplastons 對變異的甲基轉移複合酶之調節作用，而多數罹患癌症的病人就是因為這個自然防禦機轉之衰退而造成的。因此，給予 antineoplastons 之治療可以抑制腫瘤細胞生長並重建病患的自然防禦機轉。廖明徵博士從尿液中製備之 antineoplastons 混合物經證實具有抗癌作用，並發現其有效成份可能是酸性的月生月太類化合物。血液調節月生月太(Hemoregulatory Peptide, HP)是 Paukovitis 研究類似 chalone growth regulators 期間所分離鑑識出來之月生月太，其組成氨基酸序列為 pyroGlu-(Asp or Glu)-(Asp or Glu)-Cys-Lys-OH，推測為成熟之 granulocyte 所產生的，具有抑制細胞增生及調節分化之作用。因此提高月生月太抗癌作用之專一性及安定性並從事抗癌作用機轉或誘導細胞分化機轉的研究將有助於抗癌藥物之研發。本計畫選擇以 HP 之線形月生月太為先導物，從事結構活性關係之研究，並根據研究結果設計一系列之環狀月生月太衍生物以增進其安定性。針對具生物活性之環狀月生月太研發量產的反應條件以合成足夠之環狀月生月太提供構形研究之需要。研究結果有助於抗癌藥物的研發。

關鍵詞：癌症、終末細胞分化、甲基轉移複合酶、細胞增生、環狀月生月太

Abstract

Antineoplaston formulations can selectively inhibit the growth of neoplastic cells and induce the terminal differentiation of certain neoplastic cells. Mechanisms of the growth inhibition and the induction of terminal differentiation are mediated by antineoplatons,through the modulation of altered methylation complex isozymes,the malignant evolution is suppressed.It has been found that cancer patients have a breakdown of this natural defense mechanism, therefore, antineoplastons become a promising anticancer therapy to inhibit the growth of neoplastic cells and to restore the the natural defense mechanisms of the cancer patients. Antineoplastons were isolated by Liau and one of the active components was found to be acidic peptides. Hemoregulatory peptide (**HP**) was isolated and identified by Paukovitis, with the amino acid sequence pyroGlu-(Asp or Glu)-(Asp or Glu)-Cys- Lys-OH, which was produced by normal granulocyte. This peptide can regulate the differentiation pathway of haemopoiesis, therefore, modification of this peptide to increase its specificity and stability or studies of mechanism of induced cell differentiation will be useful for the development of anticancer agents.In this project we choose linear peptide **HP** as lead compound for structure activity relationship studies, and the SAR results will provide us information for further design of a series of cyclic peptide analogs to improve their stability.We will also develop the optimal conditions for large scale synthesis of active peptides for conformational studies. Results of this project will be valuable for further development of potential anticancer agents.

Keyword : cancer 、 Antineoplastons 、 terminal differentiation、methylation complex isozymes、cyclic peptide

二、緣由與目的

全世界對癌症之化學療法投注相當之心力於新藥的研發及化療作用機轉的探討，然而正統化學療法對癌症醫療效果不彰，仍無法有效的控制癌症，使得癌症的死亡率仍在持續上升。經過多年之研究，提出癌症的中心問題是甲基轉移複合酵素的異常之論點[Liau et al.]。由於甲基轉移複合酵素的異常活躍使得癌細胞無法進行終末分化，導致癌細胞不斷的分裂，此外異常的甲基轉移複合酵素會促使 DNA 過度甲基化，尤其是在 DNA 合成受到細胞毒藥物抑制的情況下 DNA 過度甲基化的情形更嚴重，而 DNA 過度甲基化是癌細胞惡化的重要因素，也是細胞毒化療失敗的原因。因此，解決癌症最有效的方法就是解決異常甲基轉移複合酵素的問題。

新鮮人尿製備的細胞分化劑 CDA-II(cell differentiation agent)可以解決異常甲基轉移複合酵素的問題，並且可以誘導癌細胞進行終末分化或凋亡[Liau et al.]。這種抗癌方法有絕對的選擇性，因此沒有不良副作用也沒有毒性，此外癌基因和抑癌基因的異常表達，轉移能力，和耐藥能力等性質也將隨著異常甲基轉移複合酵素的消失而消失。臨床測試的結果亦顯示 CDA-II 具有療效，證實細胞分化劑為解決癌症深具潛力的有效方法。雖然從尿製備的 CDA-II 有很好的療效，但是它是一種成份非常複製的混合物製劑，恐怕不易被科學先進的國家接受。最近本計畫共同主持人郭盛助教授與成大吳天賞教授積極致力於 CDA-II 成份之研究，但是迄今尚未有突破性的成果，本計畫是以合成方法企圖開發類似 CDA-II 作用之純化合物，以期獲得國際學術界之認同。

Antineoplastons 為人體及體液中自然產生之化學物質，經實驗證實可選擇性地抑制腫瘤細胞之生長並誘導腫瘤細胞進行終端細胞分化。研究發現antineoplastons抑制腫瘤細胞生長並誘導腫瘤細胞分化之反應機轉牽涉到antineoplastons 對變異的 methylation complex isozymes之調節作用，而多數罹患癌症的病人就是因為這個自然防禦機轉之衰退而造成的。因此，給予antineoplastons之治療可以抑制腫瘤細胞生長並重建病患的自然防禦機轉。尿液中製備之antineoplastons(命名為細胞分化劑 CDA-II)經證實具有抗癌作用，可選擇性地抑制腫瘤細胞之生長並誘導特定的腫瘤細胞進行終端細胞分化，並發現其有效成份可能是一些酸性的肽類化合物，但迄今仍未得到純品。

Sartorelli等研究人員發現一些實驗用之腫瘤系統可經由化學試劑之誘導使細胞分化成為成熟而無法再增生之終端細胞，因此利用誘導成熟之技術以改善腫瘤細胞之設計亦提供了治療腫瘤及研發抗癌藥物的新方向[Sartorelli, et al., 1985]。Myeloid leukemia之特性為在細胞發展相當早期的階段有成熟阻斷(maturation block)之機制, 但是一些特例中，白血病細胞可經由誘導劑之作用而克服這個限制。由罹患promyelocytic leukemia患者得來之細胞株HL-60當誘導劑加入培養液時，可誘導這類成熟之變化[Collins, et al., 1977]。Phobol ester 加入HL-60培養液可形成類似Macrophage細胞; 加入dimethyl sulfoxide, retinoic acid(RA)及其他化合物則形成類似granulocyte細胞[Harris, et al. 1985]。正常狀況下myeloid lineage之細胞增殖可直接受到下述幾種作用控制：(1) hemopoietic growth factors, 如：multi-CSF(colony stimulating factor)，granulocyte/macrophage CSF, macrophage CSF, granulocyte CSF之作用; (2) hemoregulatory peptide之抑制作用[Paukovitis, et al. 1971, 1982, 1984, Aardal, et al., 1982]。Hemoregulatory peptide(血液調節肽,HP)是Paukovitis等人研究類似chalone growth regulators期間所分離鑑識出來之肽，其組成氨基酸序列為pyroGlu-(Asp or Glu)-(Asp or Glu)-Cys-Lys-OH，推測為成熟granulocyte之產物。為了辨識出與誘導human promyelocytic HL-60 leukemia cells分化有關之作用物並且評估以誘導成熟之技術來治療白血病之概念，因此Paukovits等研究人員進一步探討變化之細胞(transformed cells,如HL-60)以retinoic acid誘導形成granulocytoid cell後是否能產生HP，結果發現產生之inhibitory peptide(抑制性肽;為了方便下述之討論本人將其簡稱為HL60P)與HP的化學及生物性質都不同。但是經由retinoic acid誘導分化之human promyelocytic HL-60 leukemia cells所產生之肽HL60P可影響haemopoiesis，而另一個由正常granulocyte產生的acidic pentapeptide HP也可以調節haemopoiesis之分化途徑。由於調節血液之肽(HP)涉及抑制細胞增生之作用，在分化過程中扮演重要的角色。因此，適當修飾肽之結構以增加其抗癌效用之專一性及其安定性將有助於抗癌藥物之研發。

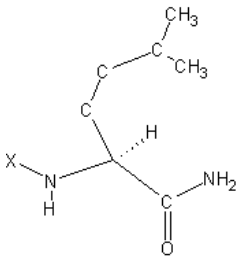
最近 HP analogs (ANT11-14)，發現這些化合物對 HL-60 細胞之 isozyme MAT^{LT} 具有很強的抑制活性(MIC 約 8 μM，尚未發表之數據)，本計畫是延續此初步的成果，繼續合成相關化合物，進而根據其結構與活性關係設計一系列之環狀肽衍生物以增進對酵素之安定性。針對具生物活性之環狀肽研發量產之反應條件，合成足夠之環狀肽以供構象研究(conformational studies)之需要。結構活性關係及構象研究的結果將有助於抗癌藥物之研發。最近郭盛助與廖明徵博士等已建立了 cancer isozyme MAT^{LT}，抑制活性，誘導 HL-60 細胞分化活性之 NBT 方法等細胞分化之篩選模式，本計畫擬合成之標的化合物，可以順利的得到活性的結果。

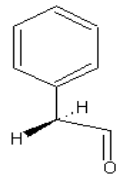
三、結果與討論

胜肽類似物之合成:

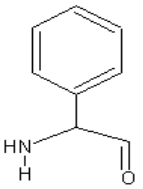
固相胜? 合成

胜? 類似物的設計是以已經發表且有效應的胜肽為藍本(圖 1). 把胜肽合成在樹脂上的技術採用現行的 standard Fmoc Chemistry (流程圖 1),合成後的胜? 從樹脂切下後經由冷凍乾燥處理再使用 RP-HPLC 進行純化. 純化後的胜肽再經由 RP-HPLC 及 FAB-Mass 定性（表 1）.

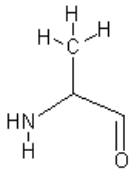




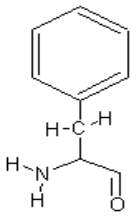
Compound **1**, X = PA, phenylacetic acid,



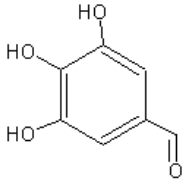
Compound **2**, X = Phg, phenylglycine,



Compound **3**, X = Ala, alanine,



Compound **4**, X = Phe,



Compound **5**, X = GA, gallic

圖 1 經過設計並合成的類胜肽（peptidomimetic）結構.

Fmoc-PAL Amide Resin

↓ Fmoc deprotection：20% piperidine/DMF

↓ Coupling：Fmoc-Leu-Resin, HOBT/HBTU/DIEA

Fmoc-Leu-Resin

↓ Fmoc deprotection：20% piperidine/DMF

↓ Coupling: X-COOH, HOBT/HBTU/DIEA

X-Leu-Resin

↓ TFA cleavage：TFA/H_2O

X-Leu-$CONH_2$

流程圖 1. 固相胜肽合成法

表 1. 合成後的胜肽的化學性質鑑定

Compounds	Peptide Sequence	HPLC Rt	FAB-MS $(M + H)^+$
1	PA-Leu-C(O)-NH_2	13.46	249 (calc. 248.3)
2	H_2N-Phg-Leu-C(O)-NH_2	14.95	264(calc. 263.3)
3	H_2N-Ala-Leu-C(O)-NH_2	6.28	183(calc. 183.2)
4	NH_2-Phe-Leu-C(O)-NH_2	7.16	260.7 (calc. 259.3)
5	GA-Leu-C(O)-NH_2	9.10	283（calc. 282.2）

生物分析

合成胜肽被用在細胞變異和細胞的分化的分析上。培養人類白血球細胞 HL-60。利用 NBT 變異分析來進行細胞變異測定。細胞的分化利用 MMT[3-（4,5-dimethylthiazol-2-yl）-2,5-diphenylterazolium bromide]分析。生物分析的結果見表二。

表二. 合成胜? **1-5** 被用在細胞變異和細胞的分化的分析

Compound	Conc. (μM)	Differentiation (%)	Proliferation (%)
Cont.	0.0	5.9 ± 0.7	100.5 ± 1.8
1. PA-Leu-C(O)-NH_2	10	2.2 ± 0.3	99.5 ± 3.4
	100	5.8 ± 1.8	62.3 ± 1.1
IC_{50}=268.0 μM	500	8.8 ± 1.5	14.4 ± 3.1
2. H_2N-Phg-Leu-C(O)-NH_2	10	1.3 ± 1.0	100.2 ± 1.2
	100	2.3 ± 0.8	95.8 ± 4.4
	500	10.5 ± 1.6	26.1 ± 2.9
IC_{50}=340.6 μM	1000	6.3 ± 0.8	49.7 ± 4.2
3. H_2N-Ala-Leu-C(O)-NH_2	10	2.5 ± 0.4	89.6 ± 3.7
	100	4.8 ± 2.0	70.1 ± 5.3
IC_{50}=236.8 μM	500	Death	6.9 ± 2.0
4.N'-Phe-Leu-C'	10	2.2 ± 0.6	114.6 ±1.7
	50	12.4 ± 3.8	28.9 ± 4.0
	100	Death	Death
IC_{50}=40.2 μM	500	Death	Death
5. N'-GA-Leu-C'	10	1.7 ± 0.8	101.5 ± 4.5
	50	6.7 ± 1.6	73.6 ± 5.0
	100	9.2 ± 2.2	15.8±3.4
IC_{50}=65.7 μM	500	Death	Death

一系列以 phenylacetic acid 為主的衍生物被合成後經由 HL-60 的細胞株測試其細胞變異的情形，我們發現這些衍生化合物比 PAA 更具有效應。其中以化合物四號最具效應。當與 PA 一起加成作用細胞之後發現化合物一號二號三號和五號更具有使的細胞變異的效應。

NBT 生物分析方法中指出若化合物與 PA 一起做測試則會增加 HL-60 細胞的變異。 MTT 生物分析方法中指出若化合物與 PA 一起做測試則會減少 HL-60 細胞的增生。

根據結果顯示 NBT 生物分析方法 PA 的相似物確實影響了 HL-60 cells 的變異。

四、計畫成果自評

1.順利成功的再實驗室中以自形設計組裝的儀器合成胜肽及類胜肽產物

2.擬發表一篇期刊論文（請參照附件一）

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附件一

Effect of N-terminal-modified Peptide Analogs on Differentiation and Proliferation of HL-60 Cells

ABSTRACT：

Phenylacetate(PA)analogs were identified as antitumor agents and found to exhibt differentiation activity in tumors. PA analogs are considered as potential antineoplastic agents. The goal of this study is to examine the structure activity relationship of PA analogs on their induction of cellular differentiation and inhibition of cellular proliferation. A series of peptides were designed on the basis of published potent analogs. Peptide analogs were synthesized by standard solid phase peptide synthesis using Fmoc chemistry. Synthetic peptides were tested against HL-60 cells for their differentiation and antiproliferation activities. Compounds **4**, **5** were found to be the most potent analogs（IC_{50}＝40.2 uM, and 65.7 uM）. Results indicate that our designed synthetic peptide analogs **4**, **5** are promising as effective cell differentiation agents.

INTRODUCTION：

Phenylacetate is a minor product of phenylalanine metabolism normally found at micromolar concentrations in the plasma and cerebrospinal fluid of humans and was identified recently as a non-toxic antitumor agent【11,8】.. Preclinical studies show that this drug and its analogs, such as phenylbutyrate, have activity of inducing the differentiation in tumors【8】. Induction of differentiation is perhaps the best approach for the systemic therapy of cancer. More importantly, clinical experience indicated that phenylacetate concentrations producing significant antitumor effects in vitro（millimolar） without significant adverse effects and well tolerated by human. The in vitro and in vivo antitumor efficacies of phenylacetate analogs have been evaluated【11】.. We study structural activity relationship of phenylacetate analogs on the differentiation and proliferation of HL-60 cells, which are the acute leukemia cells that loose the ability to differentiation into mature, functional cells and remain in a high proliferative status over their normal counterpart【17】.. The results indicate that phenylacetate analogs have the effects on inducing differentiation and inhibiting proliferation of tumors.