行政院國家科學委員會補助專題研究計畫成果報告

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※ YC-1 抑制 alpha 型腫瘤壞死因子對人類單核球誘發 ※
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行政院國家科學委員會專題研究計畫成果報告

YC-1 抑制腫瘤壞死因子對人類單核球細胞 (THP-1) 誘發第九型基質金屬酵素活性之探討

YC-1 inhibits TNF-α-induced the activation of matrix metalloproteinase-9 in THP-1 cells

計畫編號：NSC 90-2315-B-038-002

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一、中文摘要

Matrix metalloproteinases (MMPs) 為一群結構類似含鋅金屬離子之蛋白酵素，它們能夠催化分解維持組織結構之細胞外基質蛋白 (extracellular matrix proteins, ECM)，如 proteoglycans、collagen、elastin 及 laminin 等。因此對於組織之結構重組 (remodeling)、修補 (repairing) 與破壞 (destroy) 都具有相當重要之角色。同時 MMP 的含量與活性表現均受到許多方式嚴密地調節控制。根據許多文獻了解到類風濕性關節炎或血管斑塊不正常破壞且崩解基質的作用，主要源自單核球或巨噬細胞產生及釋放大量催化性基質金屬蛋白酵素。一般而言，發炎性細胞激素 (inflammatory cytokines)，如 Interleukin-1 (IL-1)、 platelet-derived growth factor (PDGF) 及 tumor necrosis factor-α (TNF-α) 均會刺激 MMP 的合成，但同時也會受生理性之內生性組織抑制劑如 TIMP-1 及 TIMP-2 所調節。

在大規模藥物篩選實驗下，我們發現其中 YC-1，一種目前已知之 soluble guanylyl cyclase (sGC) 的活化劑，具有明顯抑制 MMP 活化之作用。以電泳酵素分析法（Zymography）觀察到 YC-1 可明顯且依濃度效應（0.5-10 μM）抑制 TNF-α 誘發人類單核球細胞（THP-1 cells）之 MMP-9 活性，其抑制 50 % 反應之濃度 (IC_{50}) 為 1.0 ± 0.4 μM，而這些抑制作用又都並非源自細胞之損害。此外亦以西方墨點法 (Western blot) 明顯觀察到 MMP-9 protein 表現量隨 YC-1 劑量增加而抑制。另外，TIMP-1 protein 則無明顯影響。為了瞭解此抑制作用是否與 YC-1 促使 cGMP 量升高的作用有關，故加入 1H-(1,2,4)-oxadiazolo (4,3-a)-quinoxalin-1-one (ODQ， sGC 的抑制劑) 及 sodium nitroprusside (SNP， NO donor) 分別觀察 MMP-9 的活性是否受影響，由實驗得知並未改變原先的結果，故其抑制作用並非經由影響 cGMP 而來。

除此之外，在 RT-PCR 的實驗分析下，當給予 YC-1 (1 μM) 後亦會抑制 MMP-9 mRNA 的表現。同時我們也更進一步探討單核球細胞受 TNF-α 刺激下，細胞是否可能藉由 Nuclear factor-κB (NF-κB) 或 Mitogen-activated protein kinases (MAPKs) 活化以影響 MMP-9 之產生與活化之機轉。從結果得知 YC-1 作用後會抑制 IκB-α 的降解作用，而使 NF-κB 無法進入核中與特定的 DNA 序列接合。未來更將再進一步探討 YC-1 的抑制機轉是否經由影響 MAPKs 的活化有所相關。

關鍵詞：基質金屬蛋白酵素、YC-1、單核球細胞、nuclear factor kappa-B

Abstract

Matrix metalloproteinases (MMPs) are a family of zinc-containing proteinases, and they could degrade extracellular matrix proteins (ECM), for example, proteoglycans, collagen, elastin and laminin. Thus, it is an important role for remodeling, repairing and destroy. And the levels and activities of

MMPs are regulated and controlled by various ways. Many evidences show that human monocytes/ macrophages synthesize and secrete several MMPs which are structurally related and participate in the degradation of extracellular matrix components in either rheumatoid arthritis tissues or atherosclerotic plaques. In general, inflammatory cytokines, for example, Interleukin-1 (IL-1), platelet-derived growth factor (PDGF) and tumor necrosis factor-α (TNF-α), can stimulate the espression of MMPs, and its activity is also regulated by endogenous tissue inhibitor (TIMP-1).

According to the preliminary studies, we found that YC-1, as an activator of soluble guanylyl cyclase (sGC), could markedly attenuate MMP activation of human monocytes. YC-1 could concentration-dependently (0.5-10 μM) inhibit TNF-α-induced MMP-9 activation on human monocytes (THP-1 cells) with an IC_{50} value of 1.0 ± 0.4 μM. The inhibitory activiyies of YC-1 were not mediated by reduction of cellular viability. In addition, we found that YC-1 could concentration-dependently inhibit expression of MMP-9 protein, and without any significant effect on the expression of TIMP-1 protein. In order to understand whether the inhibitory activity of YC-1 through increasing cGMP levels, either 1H-(1,2,4)-oxadiazolo (4,3-a)-quinoxalin-1-one (ODQ, inhibitor of sGC) or sodium nitroprusside (SNP, NO donor) was tested. It was interesting that inhibitory effect of YC-1 was not abrogated by ODQ. Additionally, induction of gelatinolytic action by TNF-α was not attenuated by SNP.

Furthermore, we investigate if YC-1 affected the expression of messenger RNA (mRNA). The expression of MMP-9 mRNA was inhibited by YC-1 (1 μM) at a reduction of 70 % by the reverse transcription-polymerase chain reaction (RT-PCR). Also, we investigated the inhibitory mechanism of YC-1 on the signal transduction of TNF-α. We found that YC-1 could inhibit the degradation of IκB-α. Together, our findings revealed that YC-1 decreases MMP-9 expression in human monocytic cells through inhibition of nuclear factor kappa-B (NF-κB) activation, which may occur independent of cGMP.

Keywords: matrix metalloproteinases、YC-1、monocyte、nuclear factor kappa-B

二、緣由與目的

近年來許多文獻指出基質金屬蛋白酵素(Matrix metalloproteinses, MMPs)在正常生理具有發育之再塑作用(developmental remodeling)，亦在許多慢性發炎疾病，如類風濕性關節炎（Okada et al., 1989）、骨關節炎（Dean et al., 1989）、多發性硬化症（Ozenci et al., 1999）及粥狀動脈硬化症（Galis et al., 1994），甚至於癌症（Coussens et al., 2000）都扮演著相當重要的角色。尤其在類風濕性關節炎與粥狀動脈硬化症之發炎組織可明顯觀察到單核球(monocyte) 或巨噬細胞 (macrophage) 浸潤活化而破壞周邊結締組織（Stetler et al., 1996）。對於這種破壞細胞外基質(extracellular matrix)之不正常作用，主要是因大量的基質金屬蛋白酵素 (MMPs) 由受特定外來發炎介質（如細胞激素）所活化之單核球及巨噬細胞來產生及釋放(Woessner et al., 1991)。發炎性細胞激素(inflammatory cytokines)，如 Interleukin-1 (IL-1)、 platelet-derived growth factor (PDGF) 及 tumor necrosis factor-α (TNF-α) 均會誘使單核球進入發炎組織而影響 MMP 的含量及種類 (Redford et al., 1997； Jovanovic et al., 2000)。其中在關節炎病人之滑液檢體，可發現有大量 MMP-9 mRNA 的表現（Cawston et al., 1998）。另外在血管局部活化之基質金屬蛋白酵素可導致粥狀動脈硬化之斑塊剝離，進而導致動脈栓塞 (thrombosis)。此血管斑塊之脆弱不穩定(instability)主要源自於斑塊纖維易受損脫落與內皮細胞剝離。尤其內皮細胞剝離所造成的冠狀動脈梗塞，更可在女性及糖尿病患發生。粥狀動脈硬化形成過程中，分佈於斑塊周圍組織之巨噬細胞或其衍生之泡沫細胞與內皮細胞均可釋放並活化 MMP 以增加斑塊結締基質之不穩定性而剝損。根據臨床報告顯示剝損的發生以動脈斑塊的組成與穩定性較其體積重要得

多。因此，目前許多文獻認為，保持斑塊纖維帽的穩定完整性為預防血栓發生的重要治療方式 (Libby et al., 1995)。

近年來許多文獻顯示在腫瘤細胞轉移 (metastasis)時，會藉由細胞外基質 (extracellular matrix) 崩解的狀態下，進行侵入 (invasion) 及驅移 (chemotaxis) 反應，而造成此降解作用的酵素仍以 MMPs 為主要 (Noet et al., 1994；Crawford et al., 1996)。尤其癌細胞在轉移的過程中，其釋放的MMPs亦會導致腫瘤組織產生 (1) 血管增生 (angiogenesis) (2) 分化作用 (differentiation) (3) 增殖作用 (proliferation) 與 (4) 免疫細胞凋亡 (apoptosis) (Achim et al., 2001)。因此，MMPs 被認為是腫瘤生長擴散的重要調節因子。前述提及，MMP-2 及 MMP-9 均為 type IV collagenases，而這些酵素在多數人類腫瘤中均可被發現，其中包含肺癌、大腸癌、乳癌、血癌以及前列腺癌 (Urbanski et al., 1992；Pyke et al., 1993；Stearns et al., 1993；Lee et al., 1996；Hrabec et al., 1998)。所以近年來許多合成的 MMP 抑制劑均已被開發並開始應用於癌症病患 (Rasmussen et al., 1997)。

YC-1[3-(5'-hydroxymethyl-2'furyl)-1-benzyl-indazol] (Fig. 1)，可不經由釋放 NO 而促使 soluble guanylyl cyclase (sGC) 活化 (Ko et al., 1994)，此為有效且直接的 sGC 活化劑 (Friebe et al., 1996；Mulsch et al., 1997)。YC-1 與一般所知的 NO 及 NO donors 有相似的功能，如血小板凝集及血栓發生的抑制 (Wu et al., 1995; Teng et al, 1997)，以及血管平滑肌細胞收縮及增生的抑制 (Yu et al., 1995；Mulsch et al., 1997)。YC-1 的作用機轉可能經由多重的機制作用，其中包括 c-GMP dependent 與 independent 方式，包含了 (一) 不經由 NO 而直接活化 sGC (二) 在低濃度 NO 的存在下，cGMP 的形成 (三) 抑制 phosphodiesterase (PDE) 催化 cGMP 水解的作用 (Schmidt et al., 2001)。但無論如何，YC-1 是第一種新類型的 sGC 活化劑。

腫瘤壞死因子 (tumor necrosis factor-α, TNF-α)，主要由單核球 (monocytes) 或巨噬細胞 (macrophages) 在生理或病理因子刺激下，如 LPS、IL-1 所誘導產生。眾所皆知 TNF 的作用包含敗血性休克 (septic shock)、細胞毒性 (cytotoxicity)、發炎反應 (inflammation) 等。TNF 會刺激細胞啟動下列特殊反應，包括 (一) 誘發 nuclear factor kappa-beta (NF-κB) 活化進行基因轉錄 (gene transcription) 特定蛋白 (二) 誘發細胞凋亡 (apoptosis) (三) 經 ceramide 的脂質活化之訊息傳遞及 (四) phospholipase A_2 (PLA_2) 的活化 (Heller et al., 1994)。因此它是屬於一種多元作用性的細胞激素 (pleiotropic cytokines)，也是發炎反應中重要之作用介質，可影響許多發炎性相關蛋白質的表現 (Hu et al., 1999)。TNF-α 作用在發炎細胞，主要藉由細胞膜表面之特殊接受體 TNFR1 (p55) 或 TNFR2 (p75) (Tartaglia et al., 1992；Locksley, 2001)，再經由不同之細胞內訊息傳遞如 mitogen-activated protein kinase (MAPKs) 或 NFκB-IκB 等路徑，進而造成轉錄因子 (transcription factors) 的活化，而表現許多作用性產物。其相關轉錄因子主要以 AP-1 與 NF-κB 為主。NF-κB 由兩個屬 Rel family 類蛋白質所組成的 homo-或 hetero- 雙複合體 (dimer)，一般常見的為 p50 與 p65 的組合。對於單核球 NF-κB 的活化，主要是 TNF 接合到 TNFR1 後，使得其接合之蛋白 TRAF2 與 TRAF1 (TNF receptor associated factor, TRAF) 活化，接著使細胞內 NIK (NF-κB inducing kinase) 活化並直接催化兩種 IκB kinase 活性 (IKK-1 與 IKK-2)。此 kinase 再催化 IκB (NF-κB 抑制性蛋白) 磷酸化再經 ubiquitination 後，即被酵素複合體 (proteosome) 分解且與 NF-κB 分離，此游離態的 NF-κB 便可直接轉位 (translocation) 進入細胞核與相關之 DNA 序列接合 (如 MMP-9 之 promoter)，而增加轉錄作用速率且經 m-RNA 轉譯出大量特定作用性蛋白質 (May et al., 1998)。

Mitogen-activated protein kinase (MAPK) 屬於 serine-threonine kinase 酵素，此酵素成分為細胞對外界刺激反應作用之重要細胞內訊息介質。近來主要被研究之 MAPKs 為下列三種：(一) extracellular signal-regulated kinase (ERK1/2)、(二) c-Jun N-terminal kinase (JNK) 與 (三) p38 MAPK 等。這些相關酵素可受外來生長因子與物理性傷害等刺激因素而導致活化。較特別的是其中 JNK

及 p38 MAPK 易受如紫外線照射、熱傷害（heat shock）、高張環境（hyperosmolarity）與發炎性細胞激素（cytokines）等而活化（Kyriakis and Auruch et al., 1996）。MAPKs 在細胞訊息傳遞過程中，須自身被磷酸化才具活化之酵素型態，進而轉移（translocation）進入細胞核，且促使轉錄因子 (AP-1) 活化以進行基因表現（Roger et al., 1994）。

在各種發炎疾病狀態下，基質金屬蛋白酵素 (MMPs) 於發病過程中扮演著一個重要調節及作用的角色。如前所述，細胞激素會刺激 MMPs 的產生而造成分解結締組織，此種情況會造成各種不同之病理傷害。在本篇論文中，以人類單核球細胞 (THP-1) 為實驗細胞，藉以瞭解 YC-1 對 TNF-α 所誘發 MMP-9 protein 的活性及表現有無影響，並探討其中所牽涉的機制。更進一步我們將了解 YC-1 在 NF-κB 與 MAPK 機轉上所扮演的角色。並藉以評估將來 YC-1 在粥狀動脈硬化血管剝離及關節炎的治療可能性。

三、結果與討論

YC-1 對 TNF-α 誘發人類單核球細胞 (THP-1 cells) 產生 MMP-9 催化活性之作用

由電泳酵素分析法可得知 YC-1 隨濃度上升 (0.5、1、5 與 10 μM) 而抑制 TNF-α 所誘發 MMP-9 的活性。而其抑制百分率 (In %) 分別為 63.9 ± 9.5 (1 μM)、86.6 ± 5.0 (5 μM)、92.6 ± 3.5 (10 μM)，其抑制 50 % 反應之濃度 (IC_{50}) 為 1.0 ± 0.4 μM (n = 3-4, Fig. 2)。另外我們以 Doxycycline (50 μM, negative control) 及 PMA (16 nM, positive control) 分別進行此實驗，可發現 TNF-α 刺激 THP-1 細胞所引起之 gelatin 崩解作用，特別是 MMP-9 之分解反應，可明顯且有意義的被 Doxycycline 所抑制。在同樣實驗條件下，PMA 的確能引發大量 MMP-9 的釋放，其作用強度約為 TNF-α 的 1.5 至 2 倍 (Fig. 2)。

觀察 ODQ 及 SNP 對 TNF-α 誘發 THP-1 cells 產生 MMP-9 催化活性之作用

為了瞭解 YC-1 抑制 MMP-9 之作用是否與 YC-1 促使 cGMP 量升高的作用有關，因此我們於實驗中分別加入 ODQ (10 μM, sGC 的抑制劑) 及 SNP (10 μM, NO donor) 以觀察 MMP-9 的催化活性是否受影響。由電泳酵素分析法可得知加入 ODQ 不會使 YC-1 有意義地改變對 TNF-α 所誘發的 MMP-9 之抑制作用。單獨給予 ODQ 及 SNP 也不會抑制 TNF-α 所誘發 MMP-9 之作用。當以 resting THP-1 細胞為基準時，其 MMP-9 的活性 (gelatinolytic activity), 分別增加了 3.49 ± 0.36 (+TNF-α)、1.12 ± 0.08 (+TNF-α + YC-1)、0.96 ± 0.15 (+TNF-α + YC-1 + ODQ)、3.60 ± 0.48 (TNF-α + SNP)、2.96 ± 0.53 倍 (+PMA)。在 TNF-α 作用下，給予 YC-1 及 YC-1 + ODQ 這兩組分別與僅在 TNF-α 刺激下相比較，則為有意義的的差別 (n = 3, $p<0.01$) (Fig. 3)，且此兩組間無意義之差別。

YC-1 對 THP-1 cells 的細胞毒性

為了證明 YC-1 抑制 MMP-9 的活性及表現，並非由此成分所引發的細胞毒性，而導致 MMP-9 的含量減少。因此先將 THP-1 細胞 (2×10^5 cells/ml) 種於 24 well 的培養盤，以 YC-1 (1、5 與 10 μM) 處理細胞至 22 小時，再加入 MTT 試劑處理兩小時，利用存活細胞的粒線體內酵素將 MTT 試劑還原成 formazan 紫色結晶，再用 DMSO 將結晶溶解後測 550 nm 的吸光值，作為存活細胞的含量。由實驗結果發現 YC-1 的濃度達到 10 μM 時，也不會有意義的減少細胞存活率 (n = 3-4, Fig. 4)。

YC-1 對 THP-1 cells 以 TNF-α 誘發釋放 MMP-9 蛋白表現之作用

由 Fig. 2 已證實 TNF-α 的確能誘導產生 MMP-9 的催化作用，而 YC-1 則可將 MMP-9 活性抑制。為了瞭解 YC-1 抑制 MMP-9 的產生是否藉由減少蛋白的表現，因此我們使用西方點墨法 (Western blot) 分析 YC-1 對 MMP-9 蛋白表現的作用。由 Fig. 5 及 Fig. 6 得知，THP-1 細胞於 resting 時 (Fig. 5 及 Fig. 6, lane 1) 僅以培養液處理不同時間，分別取上清液 (supernetant) 及細胞萃取物 (lysate) 進行實驗後，發現幾乎偵測不到 92 kDa 的 MMP-9 蛋白。然而以 TNF-α 刺激 24

小時後，細胞外有大量 MMP-9 被誘導釋出 (Fig. 5, lane 2)。若以 TNF-α 刺激 12 小時後，細胞內則有大量 MMP-9 產生 (Fig. 6, lane 2)。當以 YC-1 處理後，可發現 TNF-α 引發 MMP-9 的蛋白表現隨 YC-1 的濃度增加而減少 [Fig. 5, lane 3 (0.5 μM)； lane 4 (1 μM)； lane 5 (5 μM).； Fig. 6, lane 3 (1 μM)； lane 4 (5 μM)]。

YC-1 對 TNF-α 誘發人類單核球細胞 (THP-1 cells) 釋放 TIMP-1 表現之作用

許多細胞激素如 TNF-α 均會影響 MMP-9 之活化，但同時也會受生理性之組織抑制劑如 TIMP-1 及 TIMP-2 所調節（Denhardt et al., 1993）。其中 TIMP-1 在人類單核球細胞中主要與 MMP-9 活性調節有所關聯（Leber et al., 1998）。因此我們便將實驗所取得之上清液 (supernetant) 來進行 TIMP-1 蛋白表現的實驗。同樣地，我們以西方點墨法 (Western blot) 來觀察，發現 THP-1 細胞在未經刺激時，便有些許的 TIMP-1 蛋白的表現 (Fig. 7) (lane 3)，經 TNF-α 刺激後，則可明顯見到 TIMP-1 蛋白的表現 (lane 4)。若經 YC-1 (5 μM) 處理後，則 TIMP-1 的蛋白表現僅些微受影響 (Fig. 7) (lane 5)。

YC-1 對由 TNF-α 誘導 THP-1 cells 引發 MMP-9 mRNA 之影響

萃取細胞內全部 RNA，利用反轉錄酵素 (reverse transcriptase) 將 RNA 轉換成 cDNA，於核酸循環加熱器進行聚合酵素鏈鎖反應 (簡稱 PCR) 以 MMP-9 和 GAPDH 專一性引子 (primer) 放大 MMP-9 及 GAPDH 的 cDNA 產物。以 1 % 洋菜膠 (agarose gel) 進行電泳分析。實驗結果顯示，以 TNF-α (10 nM) 處理 6 小時後，MMP-9 mRNA 明顯地被誘發出來 (Fig. 8) (lane 2)，而 YC-1 (lane 3, 1 μM)，則有意義的減少 MMP-9 mRNA 的表現，減少約 70 %。至於 housekeeping gene，GAPDH，則作為 internal control，藥物對其無影響。

YC-1 對 THP-1 cells 以 TNF-α 誘導引發 IκB 降解或 MAPK 等酵素活化的作用

依據研究指出，TNF-α 作用於發炎細胞大都經由 NF-κB-IκB 或 mitogen-activated protein kinases（MAPKs）等路徑，進而造成轉錄因子 (transcription factors) 的活化。其相關轉錄因子主要以 NF-κB 與 AP-1 為主。首先，我們利用 Western blot 來了解是否 YC-1 作用在 IκB 的磷酸化之分解過程，而影響 NF-κB 之活化。我們首先以 TNF-α 刺激觀察不同時間點 (Fig. 9) (lane 1, resting；lane 2, 5 min；lane 3, 15 min；lane 4, 30 min) 之 IκB-α 蛋白表現。實驗發現於 15 分鐘時，IκB-α 有最明顯減少，隨後又逐漸回到基準值 (basal level)。我們根據前述結果，以 TNF-α 刺激 15 分鐘進行實驗，發現以 YC-1 (Fig. 10) (lane 3, 1 μM；lane 4, 5 μM) 處理後，IκB-α 量可回到原正常表現量。因此我們推論 YC-1 可能經由抑制 IκB-α protein 的降解來減少 NF-κB 移至核內與其特定的 DNA 序列接合而達到抑制 MMP-9 的表現。另外，MAPKs 在細胞訊息傳遞過程中，須自身被磷酸化才具活化之酵素型態，進而轉移（translocation）進入細胞核，且活化轉錄因子 (AP-1) 以進行基因表現（Roger et al., 1994）。其中的 JNK 及 ERK 易受發炎性細胞激素（cytokines）而活化，所以我們未來將觀察它們在 YC-1 的抑制機轉上所扮演的角色。

綜合以上結果，研究證實 YC-1 的確可以抑制由腫瘤壞死因子 (TNF-α) 誘發人類單核球細胞 (THP-1 cells) 所釋放的 MMP-9 之活性及蛋白質表現，且此抑制效果並非來自於細胞毒性。我們亦證實 YC-1 對 MMP-9 降解細胞外基質的抑制能力，且與 YC-1 可促使 cGMP 量升高的作用無關，也與內生性組織抑制劑如 TIMP-1 的作用無關。另外，我們亦證實 YC-1 的抑制效果可能經由 NF-κB 此步驟來達成。因此，未來我們將針對細胞訊息相關的 JNK 及 ERK 路徑以探討其是否有其它的抑制機轉。藉此初步結果來評估 YC-1 能否作為將來慢性發炎疾病如粥狀動脈硬化血管剝離與類風濕性關節炎治療的可能性。

四、參考文獻

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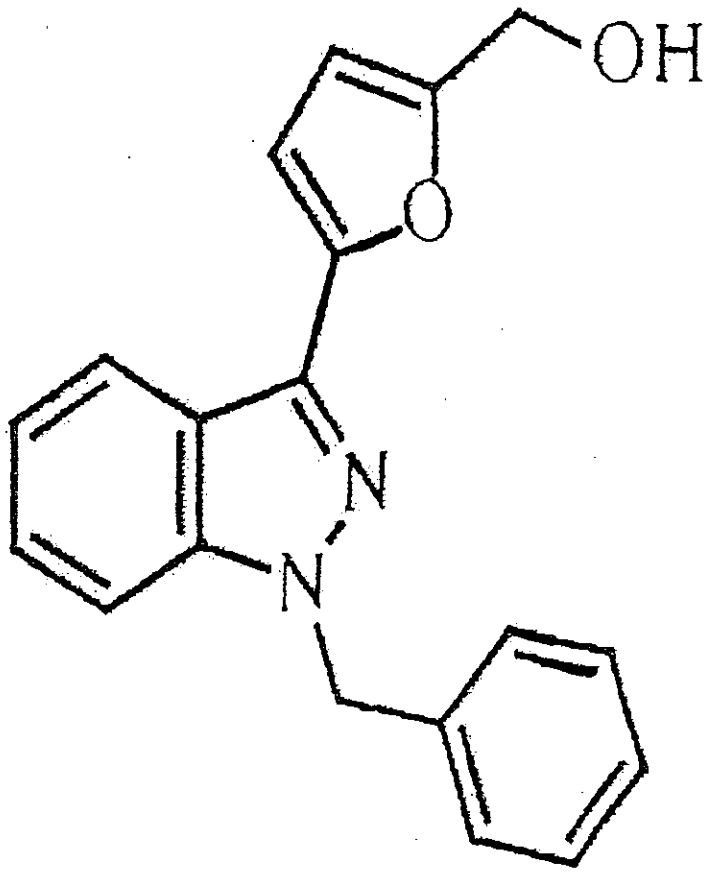
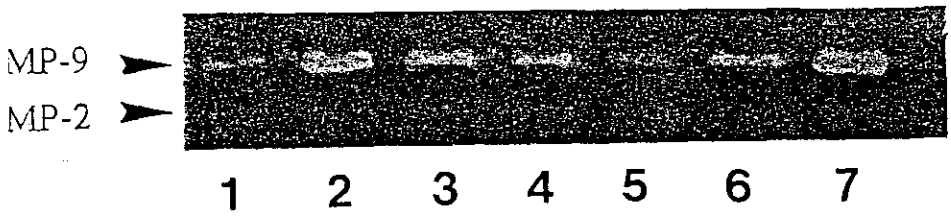


Figure 1. Chemical structures of YC-1 【3-（5'-hydroxymethyl-2'furyl）-1-benzyl-indazol】



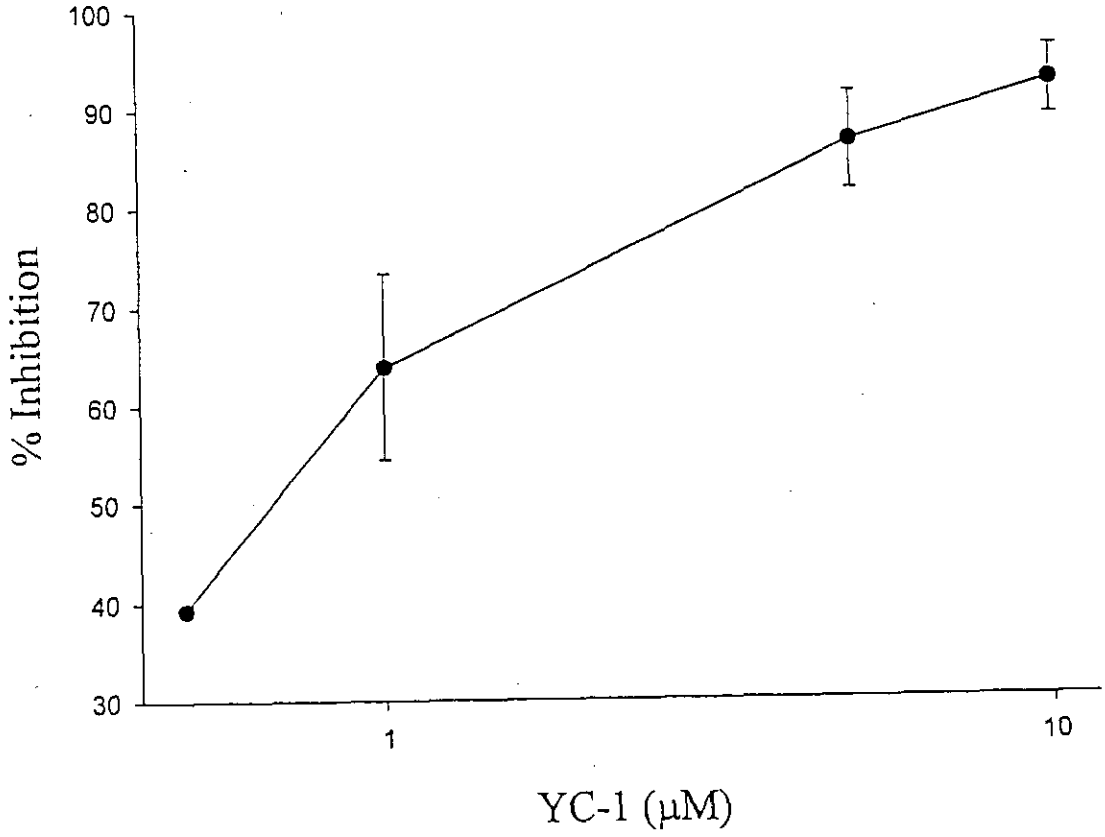


Figure 2. Effect of YC-1 on TNF-α-induced enzymatic activity of matrix metalloproteinase-9 (MMP-9) in THP-1 cells. THP-1 cells (2×10^6 cell/ml) were dispensed on 24-well plates till 70-80 % confluent condition and treated with TNF-α (10 nM) for 24 hrs as indicated. Cells were treated with the indicated concentrations of YC-1 (lane 3, 0.5 μM ; lane 4, 1 μM ; lane 5, 5 μM) or vehicle (DMSO, 0.25 % v/v, lane 2 ; Doxycycline, 50 μM, as negative control, lane 6) for 15 min before treatment with TNF-α. Cell-free supernatants were then assayed for MMP-9 activity by gelatin zymography, as detailed in " Methods " (lane 1: control ; lane 7: PMA, 16 nM, as positive control). Percent inhibition is presented as mean ± S.E.M. of three to five independent experiments.

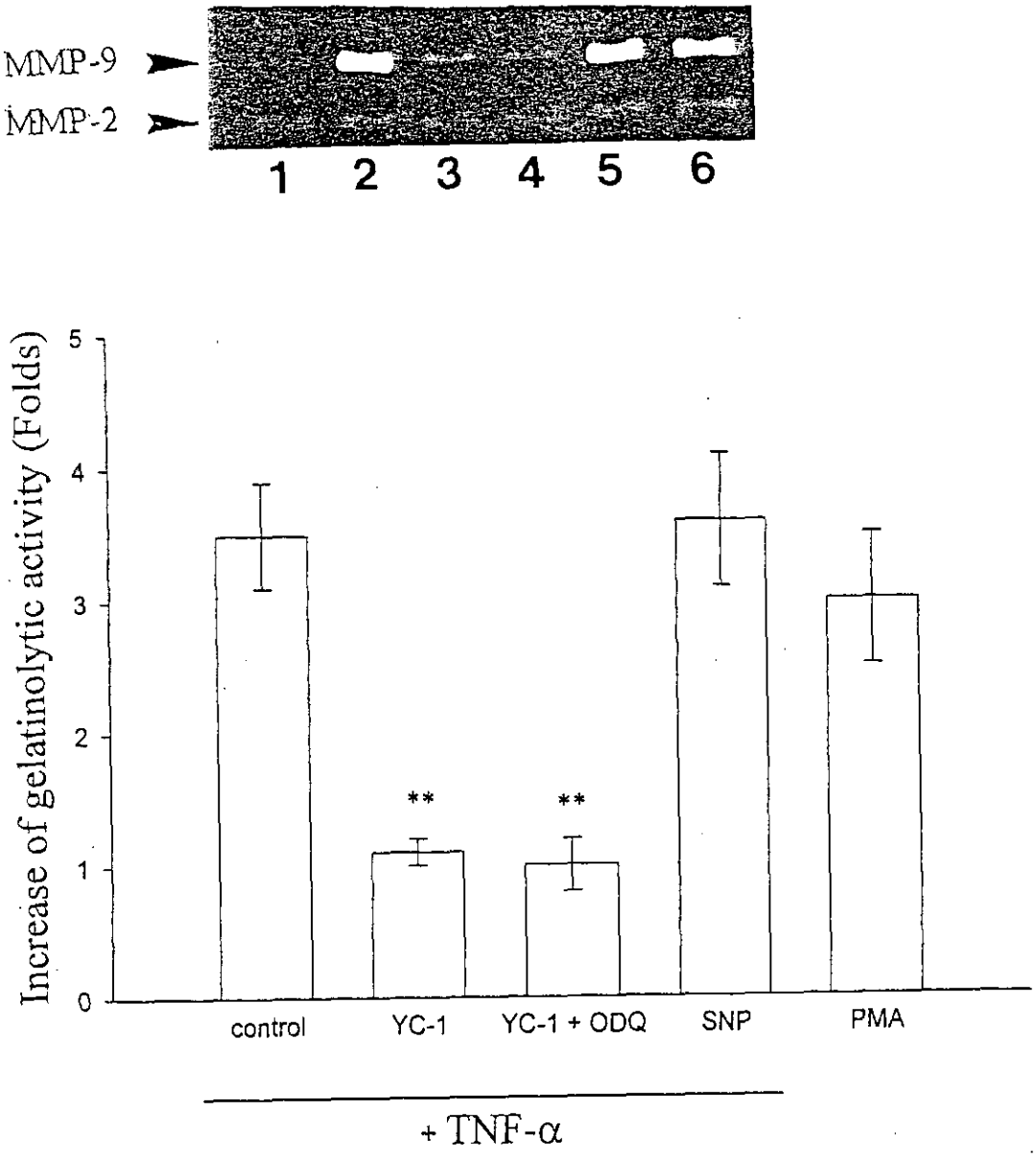


Figure 3. Effects of ODQ and SNP on TNF-α-induced activity of matrix metalloproteinase-9 (MMP-9) in THP-1 cells. THP-1 cells (2×10^6 cell/ml) were dispensed on 24-well plates till 70-80 % confluent condition and treated with TNF-α (10 nM) for 24 hrs as indicated. Cells were treated with YC-1 (5 μM), ODQ (10 μM) and SNP (10 μM) for 15 min before treatment with TNF-α. Cell-free supernatants were then assayed for MMP-9 activity by gelatin zymography, as detailed in " Methods " (lane 1: resting ; lane 2: TNF-α ; lane 3 : TNF-α + YC-1 ; lane 4 : TNF-α + YC-1 + ODQ ; lane 5 : TNF-α + SNP ; lane 6 : PMA,16 nM, as positive control). Folds of the increase of gelatinolytic activity are presented as mean ± S.E.M. of three independent experiments. ** : $p < 0.01$ as compared with the control.

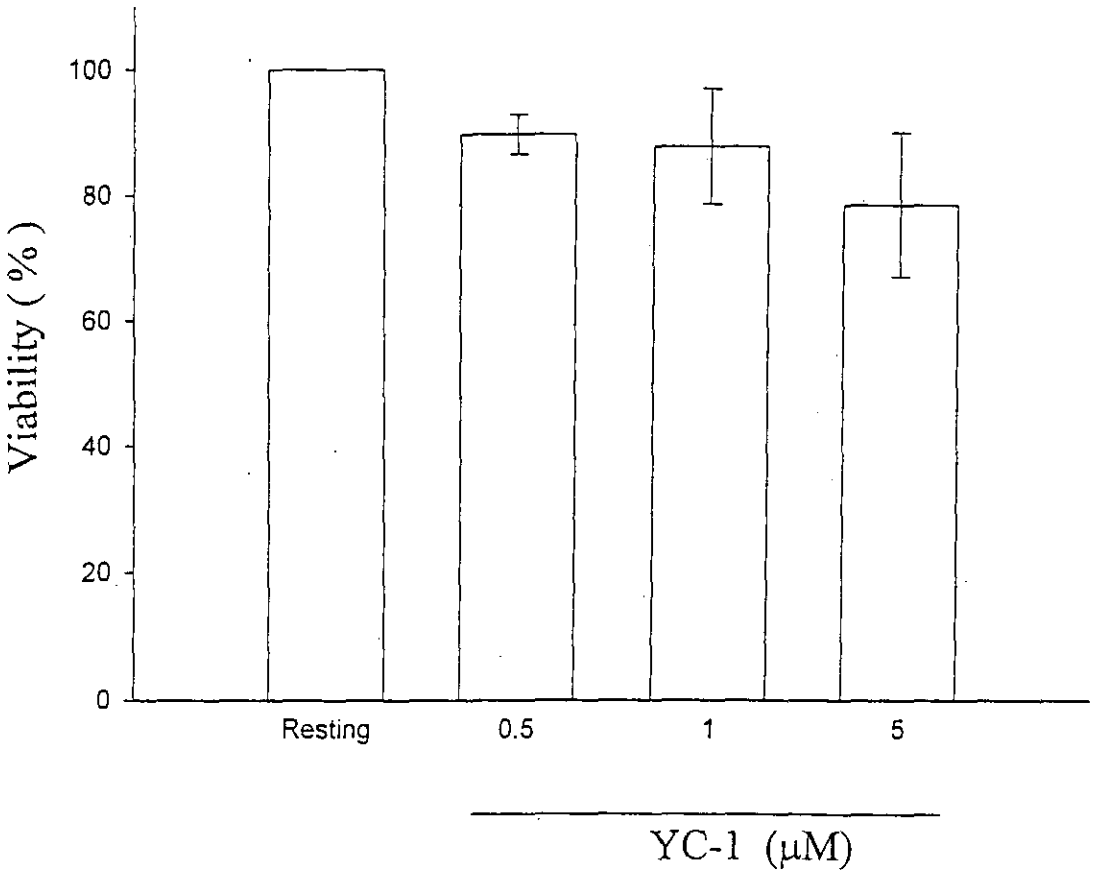


Figure 4. Cytotoxicity of YC-1 on THP-1 cells. THP-1 cells were treated with different concentrations of YC-1 (0.5, 1, 5 μM) and incubated for 24 hrs. Cell viability was measured by a colorimetric assay at 550nm based on the ability of mitochondria to reduce the tetrazolium dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) in viable cells. Percent cell viability was calculated as: (absorbance of treated cells) / (absorbance of control cells) × 100 %. Percentage of viability is presented as mean ± S.E.M. of three to four independent experiments.

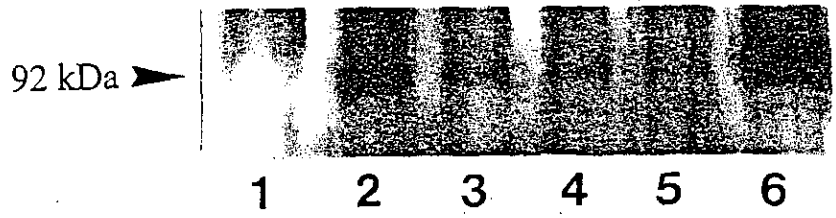


Figure 5. Effect of YC-1 on TNF-α-induced production of matrix metalloproteinase-9 (MMP-9) from conditioned medium of THP-1 cells. THP-1 cells (2×10^6 cell/ml) were dispensed on 24 well plate till 70-80 % confluent condition and treated with YC-1 (lane 3, 0.5 μM; lane 4, 1 μM; lane5, 5 μM) or vehicle (DMSO, 0.25 % v/v, lane 2) for 15 min before treatment with TNF-α (10 nM) for 24 hrs. Then supernatants were obtained and analysed for MMP-9 protein expression by Western blot (lane 1, resting, lane 6, PMA, 16 nM). The data are representative example of three experiments.

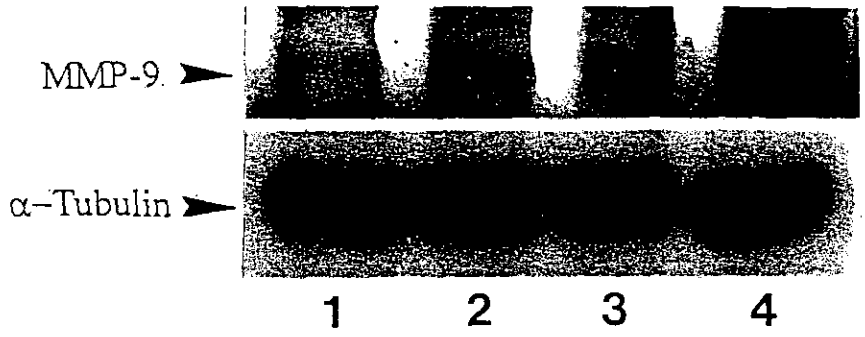


Figure 6. Effect of YC-1 on TNF-α-induced protein expression of matrix metalloproteinase-9 (MMP-9) in THP-1 cells. THP-1 cells (1×10^6 cell/ml) were dispensed on 6 well plate till 70-80 % confluent condition and treated with YC-1 (lane 3, 1 μM; lane 4, 5 μM) or vehicle (DMSO, 0.25 % v/v, lane 2) for 15 min before treatment with TNF-α (10 nM) for 12 hrs. Then whole-cell lysates were analysed for MMP-9 protein expression by Western blot (lane 1, resting without TNF-α). α–Tubulin (57 kDa) shown as an internal control. The data are representative example of three experiments.

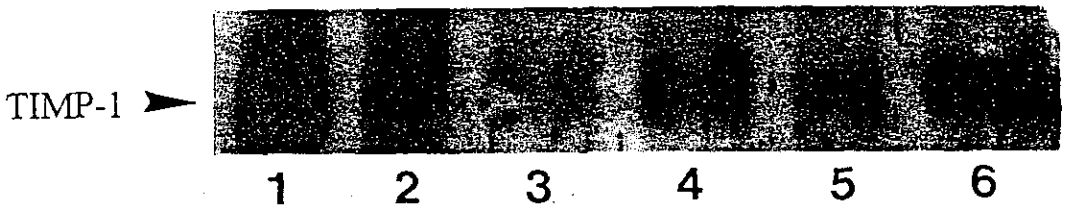


Figure 7. Effect of YC-1 on tissue inhibitors of metalloproteinase (TIMPs) from conditioned medium of THP-1 cells. THP-1 cells (2×10^6 cell/ml) were dispensed on 24 well plate till 70-80 % confluent condition and treated with YC-1 (lane 5, 5 μM) or vehicle (DMSO, 0.25 % v/v, lane 4) for 15 min. And cells were incubation with TNF-α (10 nM) for 24 hrs. Then supernatants were obtained and analysed for TIMP-1 protein expression by Western blot (lane 1, positive control; lane 2, marker; lane 3, resting without TNF-α; lane 6, PMA 16nM). The data are representative example of three experiments.

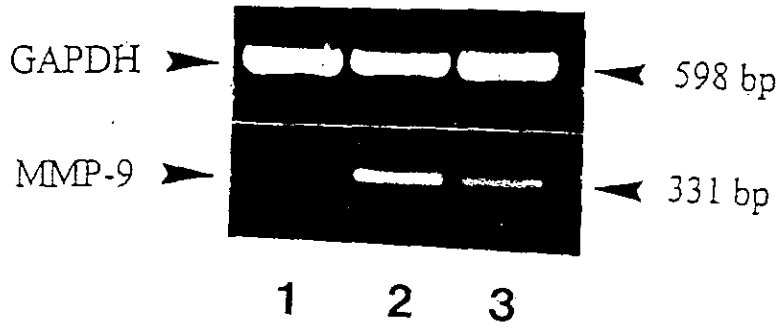


Figure 8. RT-PCR analysis demonstrating the effect of YC-1 on TNF-α-induced MMP-9 mRNA expression in THP-1 cells. Cells were treated with YC-1 (lane 3, 1 μM) or vehicle (DMSO, 0.25 % v/v, lane 2) for 15 min before treatment with TNF-α (10 nM) for 6 hrs. Following by extraction of total RNA and analysis of mRNA levels of MMP-9 and GAPDH. RT-PCR technique was performed as described in "Methods" (lane 1, resting without TNF-α). GAPDH levels normalized the amount of cDNA temple used in each PCR reaction.

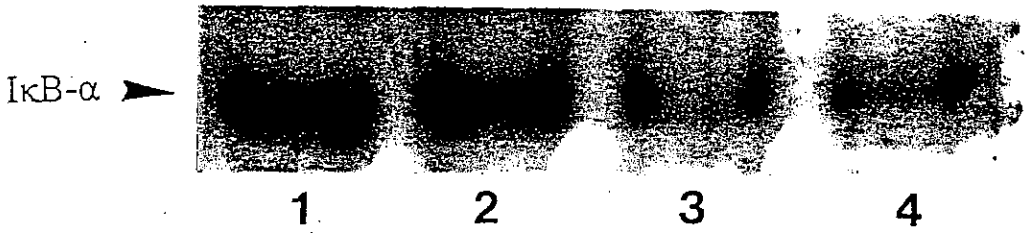


Figure 9. Western blot analysis demonstrating the time course on degradation of immunoreactive IκB-α in THP-1 cells (1×10^6 cell/ml). THP-1 cells were dispensed on 6-well plate till 70-80 % confluent condition and treated with TNF-α (lane 2, 5 min ; lane 3, 15 min ; lane 4, 30 min) or vehicle (DMSO, 0.25 % v/v, lane 1) as indicated. The data are representative example of three experiments.

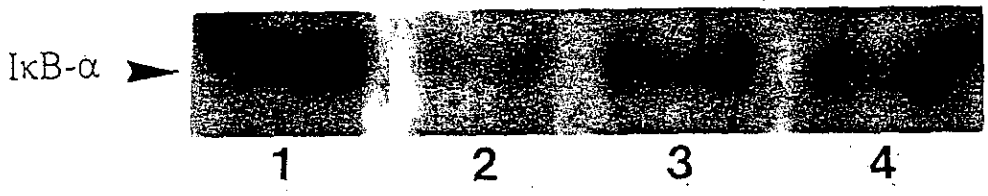


Figure 10. Effect of YC-1 on degradation of immunoreactive IκB-α in THP-1 cells. THP-1 cells (1×10^6 cells/ml) were dispensed on 6-well plate till 70-80 % confluent condition and treated with TNF-α (10 nM) for 15 min as indicated. Cells were treated with YC-1 (lane 3, 1 μM ; lane 4, 5 μM) or vehicle (DMSO, 0.25 % v/v, lane 2) for 15 min before treatment with TNF-α. Then cells were obtained and analysed for IκB-α protein expression by Western blot (lane 1, resting without TNF-α). The data are representative example of three experiments.