行政院國家科學委員會專題研究計畫　成果報告

連接 vasostatin 和腫瘤抗原的去氧核糖核酸疫苗其抗腫瘤效用和作用機轉

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-002-377-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立臺灣大學醫學院婦產科

計畫主持人：鄭文芳

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行政院國家科學委員會補助專題研究計畫 **■ 成 果 報 告**
□期中進度報告

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成果報告類型(依經費核定清單規定繳交)：□精簡報告　▒完整報告

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執行單位：國立臺灣大學醫學院婦產科

中　華　民　國　　年　　月　　日

一、中文摘要

關鍵詞：

理想治療癌症的方向必須可以清除身體上多處發生的腫瘤，同時可以明確地區分腫瘤細胞和非腫瘤細胞。以此觀點來看抗癌特異的癌症免疫治療代表了一個吸引人的腫瘤療法。活化抗原特異 T 細胞調節的免疫反應並且殺死帶有特異抗原的腫瘤細胞。最近我們將 Calreticulin 鍵結 HPV16 的 E7 並發現 CRT/E7 DNA 疫苗表現出提高 E7 的 CD8+T 先驅細胞和抗 E7 表達腫瘤顯著地抗癌效果，利用 Calreticulin 鍵結腫瘤抗原顯示令人興奮的抗癌效果。

在這個研究，我們發現 C57BL/6 鼷鼠接受 Calreticulin 的 N-domin 鍵結 E7 的 DNA 疫苗也顯出可增加 E7 特異 CD8+T 先驅細胞的數目和顯著地抗癌效果。NCRT/E7 和 CRT/E7 兩者抗癌效果相似，但 CRT/E7 可以產生較多數目的 E7 特異 CD8+T 先驅細胞。NCRT/E7 鍵結腫瘤抗原是令一個有潛力做為抗原特異免疫治療的分子。

二、英文摘要

The ideal cancer treatment should be able to eradicate systemic tumors at multiple sites in the body while having the specificity to discriminate between neoplastic and nonneoplastic cells. In this regard, antigen-specific cancer immunotherapy represents an attractive approach for cancer treatment. Activation of antigen-specific T cell–mediated immune response allows to kill tumors associated with a specific antigen. Recently, we linked careticulin (CRT) to HPV16 E7 and found that CRT/E7 DNA exhibited a dramatic increase in E7-specific CD8+ T cell precursors and an impressive antitumor effect against E7-expressing tumors. Cancer therapy using CRT linked to a tumor antigen holds promise for treating tumors by combining antigen-specific immunotherapy and antiangiogenesis [1].

In this study, we found that C57BL/6 mice vaccinated intradermally with N-domain of calreticulin (NCRT) linked with E7 (NCRT/E7) DNA also exhibited dramatic increases in E7-specific CD8+ T cell precursors and impressive antitumor effects against E7-expressing tumors compared with mice vaccinated with wild-type E7 DNA. As comparing with CRT/E7, NCRT/E7 can generate similar anti-tumor effect. However, CRT/E7 DNA vaccine can generate higher numbers of E7-specific CD8 T precursors rather than NCRT/E7 does. Thus, cancer therapy using NCRT linked to a tumor antigen which is another promising molecule for antigen-specific immunotherapy.

三、計畫原由與目的

The ideal cancer treatment should be able to eradicate systemic tumors at multiple sites in the body while having the specificity to discriminate between neoplastic and nonneoplastic cells. In this regard, antigen-specific cancer immunotherapy and antiangiogenesis represent two attractive approaches for cancer treatment. Activation of antigen-specific T cell–mediated immune responses allows for killing of tumors associated

with a specific antigen [2, 3] while inhibition of angiogenesis controls neoplastic growth by sequestering neoplastic cells from an adequate blood supply [4, 5]. Therefore, an innovative approach that combines both mechanisms will likely generate the most potent antitumor effect.

The use of calreticulin (CRT) represents a feasible approach for enhancing tumor-specific T cell–mediated immune responses. CRT is an abundant 46 kDa Ca2+-binding protein located in the endoplasmic reticulum (ER) [6]. The protein has been shown to associate with peptides delivered into the ER by transporters associated with antigen processing (TAP-1 and TAP-2) [7] and with MHC class I-β2 microglobulin molecules to aid in antigen presentation [8]. Previous studies have shown that CRT can be complexed with peptides *in vitro* to elicit peptide-specific CD8+ T cell responses through exogenous administration [9]. The protein is composed by three domains. There are N-domain, P-domain and C-domain. The N-domain (residues1–180) is the most conserved domain among calreticulins from different species [10]. The P-domain (residues 181–280) is rich in proline and contains two sets of three sequence repeats. The C-domain of calreticulin is reminiscent of the C-terminal region of calsequestrin of muscle sarcoplasmic reticulum. The C-domain terminates with the KDEL ER retrieval signal.

In the present study, we examined could the N domain of calreticulin (NCRT) when links with a tumor antigen can also generate the antitumor activities. Therefore, cancer vaccines employing N-domain of calreticulin, may potentially generate antitumor effects by enhancing antitumor immune responses. We investigated the novel use of N-domian of calreticulin chimerically linked to a model antigen (HPV-16 E7) for the treatment of tumors through the combination of antigen-specific immunotherapy. We found that C57BL/6 mice vaccinated intradermally with NCRT/E7 DNA exhibited dramatic increases in E7-specific CD8+ T cell precursors and impressive antitumor effects against E7-expressing tumors compared with mice vaccinated with wild-type E7 DNA. We also found that NCRT/E7 can generate similar anti-tumor effect when compared with CRT/E7. The CRT/E7 DNA vaccine generates a higher number of E7-specific CD8 T precursors than NCRT/E7 in the immunologic assays. Thus, cancer therapy using NCRT linked to a tumor antigen holds a promising way for treating tumors by antigen-specific immunotherapy.

四、實驗方法

Plasmid DNA Constructs and Preparation

The generation of pcDNA3-E7 has been described previously [11, 12]. The generation of pcDNA3-CRT has also been described previously [1]. For the generation of N-domain of CRT, NCRT were first amplified with PCR by using rabbit CRT cDNA as the template [13] and a set of primers, 5’-CCGGTCTAGAACGCTGCTCCCTGTGCCGCT-3’ and 5’-CCCGAATTCGTTGTCCGGGCCGCACGATCA-3’.

There is more than 90% homology between rabbit, human, mouse, and rat CRT [14].

Then, the amplified product of NCRT was cloned into the XbaI/EcoRI sites of pcDNA3 vector (Invitrogen Corp., Carlsbad, California, USA). For the generation of pcDNA3-NCRT/E7, E7 was first amplified with a set of primers, 5'-ggggaattcatggagatacaccta-3'和and 5'-ggtggatccttgagaacagatgg-3', and then cloned it into the EcoRI/BamHI sites of pcDNA3-NCRT to generate pcDNA3-CRT/E7. Plasmid constructs were confirmed by DNA sequencing.

Cell Line

The production and maintenance of TC-1 cells have been described previously [15].

DNA Vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination were performed using a helium-driven gene gun (BioRad Laboratories Inc., Hercules, California, USA) according to a protocol described previously [16]. Control plasmid, E7, NCRT, NCRT/E7 or CRT/E7 DNA-coated gold particles was delivered to the shaved abdominal region of mice using a helium-driven gene gun (BioRad Laboratories Inc.) with a discharge pressure of 400 psi.

Intracellular Cytokine Staining and Flow Cytometry Analysis

Mice were immunized with 2 μg of the various DNA vaccines and received a booster with the same regimen 1 week later. Splenocytes were harvested 1 week after the last vaccination. Before intracellular cytokine staining, $3.5x10^5$ pooled splenocytes from each vaccination group were incubated for 16 hours with either 1 μg/ml of E7 peptide (aa 49-57) containing an MHC class I epitope [17] for detecting E7-specific CD8+ T cell precursors or 10 μg/ml of E7 peptide (aa 30-67) containing an MHC class II epitope [18] for detecting E7-specific CD4+ T cell precursors. Cell surface marker staining for CD8 or CD4 and intracellular cytokine staining for IFN-γ, as well as flow cytometry analysis, were performed by using conditions described previously [19].

Enzyme-linked Immunoabsorbent Assay (ELISA) for Anti-E7 Antibody

For the detection of HPV 16 E7-specific antibodies in the sera, a direct ELISA was used as described previously [20]. Mice were immunized with 2 μg of the various DNA vaccines and received a booster with the same regimen 1 week later. Sera were prepared from mice on day 14 after immunization. Briefly, a 96-microwell plate was coated with 100 μl of bacteria-derived HPV-16 E7 proteins (0.5 μg/ml) and incubated at 4°C overnight. The wells were then blocked with phosphate-buffered saline (PBS) containing 20% fetal bovine serum. Sera were prepared from mice on day 21 postimmunization, serially diluted in PBS, added to the ELISA wells, and incubated at 37°C for 2 hr. After washing with PBS containing 0.05% Tween 20, the plate was incubated with a 1:2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for 1 hr. The plate was washed, developed with 1-Step Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1 *M* H_2SO4. The ELISA plate was read with a

standard ELISA reader at 450 nm.

ELISPOT Assay

The ELISPOT assay described by Miyahira *et al.* [21] and Murali-Krishna *et al.* [22] was modified to detect HPV-16 E7-specific CD8+ T cells. The 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml rat antimouse IFN-γ antibody (clone R4–6A2, PharMingen, San Diego, CA) in 50 μl of PBS. After overnight incubated at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. Different concentrations of fresh isolated spleen cells from each vaccinated mice group, starting from 1 x 10^6/well, were added to the well along with 15 IU/ml IL-2. Cells were incubated at 37°C for 24 h either with or without 1 μg/ml E7-specific H-2D^b CTL epitope (E7, amino acids 49–57). After culture, the plate was washed and then followed by incubating with 5 μg/ml biotinylated IFN-γ antibody (clone XMG1.2, PharMingen) in 50 μl in PBS at 4°C overnight. After washing six times, 1.25 μg/ml avidin-alkaline phosphatase (Sigma, St. Louis, MO) in 50 μl of PBS were added and incubated for 2 h at room temperature. After washing, spots were developed by adding 50 μl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Boehringer Mannheim, Indianapolis, IN) and incubated at room temperature for 20 minutes. The spots were counted by using a dissecting microscope.

***In Vivo* Tumor Protection Experiments**

For the tumor protection experiment, C57BL/6 mice (five per group) either received no vaccination or were immunized with 2 μg/mouse of plasmid without insert, NCRT, E7, NCRT/E7 or NCRT mixed with E7 (NCRT+E7) DNA with a gene gun. One week later, mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were subcutaneously challenged with 5xl0^4 TC-1 cells/mouse in the right leg. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week until they were sacrificed at day 60.

***In Vivo* Ab Depletion Experiments**

In vivo Ab depletions were performed as described previously [23]. Briefly, C57BL/6 mice (five per group) were vaccinated with 2 μg/mouse of NCRT/E7 DNA with a gene gun, boosted 1 week later, and challenged with 5x10^4 cells/mouse TC-1 tumor cells. Depletion was started 1 week before tumor challenge. The mAb GK1.5 was used for CD4 depletion [24], mAb 2.43 was used for CD8 depletion [25], and mAb PK136 was used for NK1.1 depletion [26]. Depletion was terminated on day 40 after tumor challenge.

五、結果與討論

Vaccination with NCRT/E7 DNA significantly enhances E7-specific CD8+ T cell–mediated immune response

As shown in **Figure 1A and 1B**, vaccination with NCRT/E7 DNA generated the higher frequency of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors (169.0/3.5x10^5

splenocytes) compared with mice vaccinated with E7 DNA (12.0/3.5x10^5 splenocytes) ($p<0.01$). Our results also indicated that the fusion of NCRT to E7 was required for enhancement of $CD8^+$ T cell activity, since NCRT DNA mixed with E7 DNA did not generate enhancement of $CD8^+$ T cell activity (data not shown). We further evaluated that if the NCRT/E7 could also enhance the E7-specific CD4+ T cell response. We observed no increase in the number of E7-specific IFN-γ-secreting $CD4^+$ T cells in mice vaccinated with NCRT/E7 DNA compared with mice vaccinated with control plasmid, E7, NCRT, or NCRT+E7 DNA **(Figure 1C)**.

We further performed ELISPOT assays to evaluate the enhancement of E7-specific immunologic response of NCRT/E7 DNA vaccine. As shown in **Figure 2**, 366 IFN-γ spot-forming CD8+ T cells specific for the immunodominant Db-restricted E7 peptide were detected per 10^6 splenocytes derived from the NCRT/E7 DNA vaccinated mice, compared to only 8 E7-specific IFN-γ spot-forming CD8+ T cells/10^6 splenocytes derived from the E7 DNA-vaccinated mice.

Vaccination with NCRT/E7 DNA significantly enhances E7-specific Ab response

To determine if NCRT/E7 DNA could enhance E7-specific antibody immune responses in mice, we performed ELISA experiments. As shown in **Figure 3**, mice vaccinated with NCRT/E7 or CRT/E7 DNA generated significantly higher titers of anti-E7 Ab's in the sera of mice compared with the other vaccinated groups ($P<0.01$). Our results indicated that the fusion of NCRT to E7 could enhance the E7-specific Ab response and the titer of E7-specific Ab generated from NCRT/E7 DNA vaccine is as high as that from CRT/E7 DNA vaccine.

Vaccination with NCRT/E7 DNA enhances tumor protection in mice challenged with an E7-expressing tumor cell line

To determine if the observed enhancement in E7-specific $CD8^+$ T cell–mediated immunity translated to a significant E7-specific antitumor effect, we performed an *in vivo* tumor protection experiment using a previously characterized E7-expressing tumor model, TC-1 [15]. As shown in **Figure 4**, 100% of mice receiving NCRT/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. In contrast, all of the unvaccinated mice and mice receiving plasmid without insert, NCRT, wild-type E7, or NCRT mixed with wild-type E7 DNA developed tumors within 15 days after tumor challenge. Our results also indicated that the fusion of NCRT to E7 was required for antitumor immunity, since NCRT mixed with E7 (NCRT+E7 DNA) did not generate significant enhancement of antitumor immunity in the preventive experiments.

$CD8^+$ T cells but not $CD4^+$ T cells or natural killer cells are essential for the antitumor effect generated by NCRT/E7 DNA

To determine the subset of lymphocytes important for the antitumor effect, we performed in vivo Ab depletion experiments [27]. As shown in **Figure 5**, all naïve mice

and all mice depleted of $CD8^+$ T cells grew tumors within 15 days after tumor challenge. In contrast, all of the nondepleted mice and all of the mice depleted of $CD4^+$ T cells or NK1.1 cells remained tumor-free 60 days after tumor challenge. These results suggested that $CD8^+$ T cells are important for the antitumor immunity generated by the NCRT/E7 DNA vaccine.

In this study, we demonstrated that linkage of HPV-16 E7 antigen to NCRT can significantly enhance the potency of an E7-expressing DNA vaccine. The N-domain of calreticulin linked with E7 DNA elicited strong E7-specific CD8+ T cell immune responses, generated significant $CD8^+$ T cell–dependent protective effects against subcutaneous HPV-16 E7-expressing tumors. Thus, DNA vaccines encoding NCRT chimerically linked to a tumor antigen represent an immunological approach for the generation of a potent antitumor effect.

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七、圖解

Figure 1. Flow cytometry analysis of IFN-γ-secreting E7-specific $CD8^+$ and $CD4^+$ T cell precursors in mice vaccinated with various recombinant DNA vaccines. Mice were vaccinated via gene gun with pcDNA3 with no insert, E7, NCRT, NCRT/E7, PCRT/E7, CCRT/E7 and CRT/E7 DNA. One week later, mice were boosted with the same regimen as the first vaccination. Splenocytes from naïve or vaccinated mice were cultured in vitro with 1 μg/ml of E7 peptide (aa 49–57) or 10 μg/ml of E7 peptide (aa 30-67) overnight and analyzed for both CD8 or CD4 and intracellular IFN-γ by flow cytometry analysis. **(A) Representative figures of FACScan analysis. (B) Number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors.** Data are expressed as mean number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors per $3.5x10^5$ splenocytes ± SEM. *Note:* Mice vaccinated with NCRT/E7, PCRT/E7 or CCRT/E7 DNA generated the higher number of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors as compared with the wild-type E7 group did. However, the numbers of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors in the NCRT/E7, PCRT/E7 and CCRT/E7 groups weres lower than those in CRT/E7 group. **(C) Number of E7-specific IFN-γ-secreting $CD4^+$ T cell precursors.** Data are expressed as mean number of E7-specific IFN-γ-secreting $CD4^+$ T cell precursors per $3.5x10^5$ splenocytes ± SEM. ***Note:*** Mice vaccinated with NCRT/E7 DNA did not generate the higher number of E7-specific IFN-γ-secreting $CD4^+$ T cell precursors as compared with the other vaccination groups.

Figure 2. The representative figures of ELISPOT analysis of E7-specific $CD8^+$ T cell precursors in mice vaccinated with various recombinant DNA vaccines. The ELISPOT assays were performed to evaluate the E7-specific CD8+ T cells as described in **Materials and Methods**. Mice were vaccinated with various DNA vaccines and the splenocytes were collected and treated as described in **Figure Legend 1**. (1) Naïve group (2) No insert group (3) wild-type E7 group (4) NCRT group (5) NCRT/E7 group. *Note*: Mice vac**c**inated with NCRT/E7 DNA generated the higher number of IFN-γ+ spots when compared to wild-type E7 group.

Figure 3. E7-specific antibody responses in mice immunized with various naked DNA vaccines. The presence of E7-specific antibodies was detected by ELISA, using serial dilutions of serum. The results from the 1:100, 1:500, and 1:1,000 dilution are presented, showing mean absorbance (OD) at 450 nm ± SE. **Titers of E7-specific antibodies in**

naïve mice or mice vaccinated with pcDNA3 with no insert, E7, NCRT and NCRT/E7.
Note: NCRT/E7 DNA vaccine generated significantly higher E7-specific antibody responses when compared with mice vaccinated with the other DNA vaccines ($P<0.01$, one-way ANOVA).

Figure 4. *In vivo* tumor protection experiments in mice vaccinated with various DNA vaccines. Mice were immunized with various DNA vaccines and challenged as described in **Materials and Methods** to assess the antitumor effect generated by each DNA vaccine. *Note:* 100% of mice receiving NCRT/E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. All of the unvaccinated mice and mice receiving plasmid without insert, NCRT, wild-type E7, or NCRT mixed with wild-type E7 DNA developed tumors within 15 days after tumor challenge.

Figure 5. *In vivo* Ab depletion experiments. Mice were immunized with NCRT/E7 DNA and challenged with TC-1 tumor cells to determine the effect of lymphocyte subsets on the potency of the NCRT/E7 DNA vaccine. CD4, CD8, and NK1.1 depletions were initiated 1 week before tumor challenge and lasted 40 days after tumor challenge. *Note:* All naïve mice and all mice depleted of $CD8^+$ T cells grew tumors within 15 days after tumor challenge. All of the nondepleted mice and all of the mice depleted of $CD4^+$ T cells or NK1.1 cells remained tumor-free 60 days after tumor challenge.

八、圖示

Figure 1

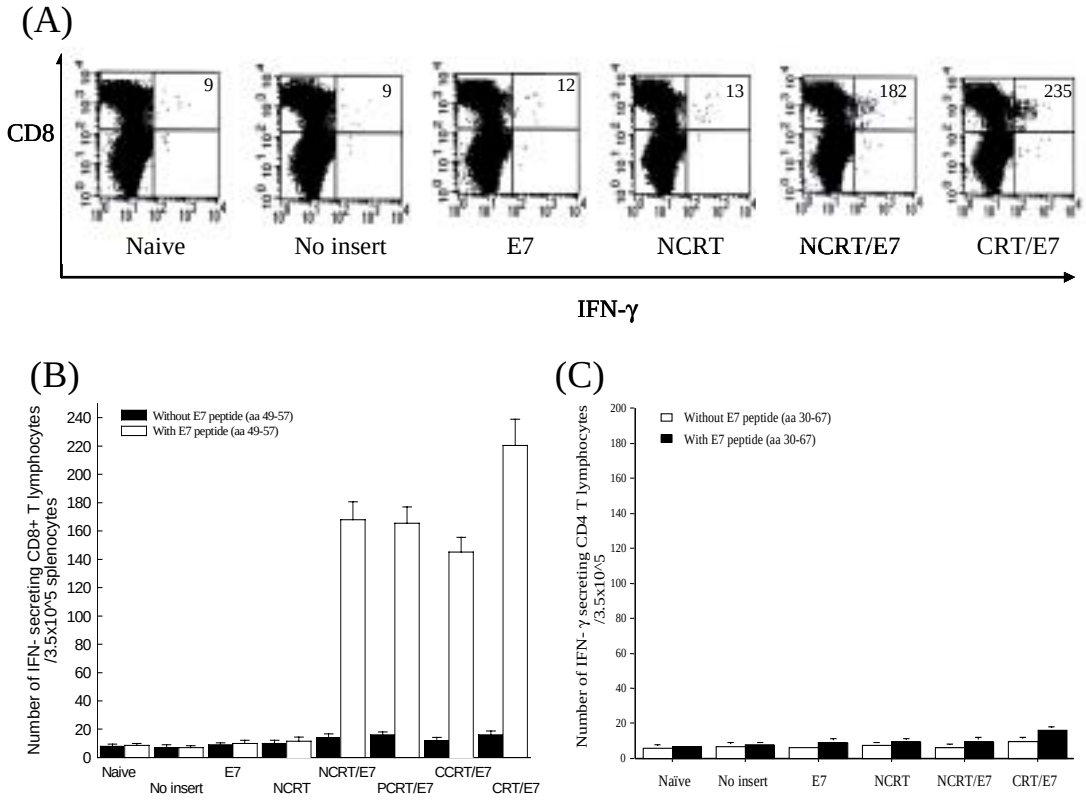

Figure 2

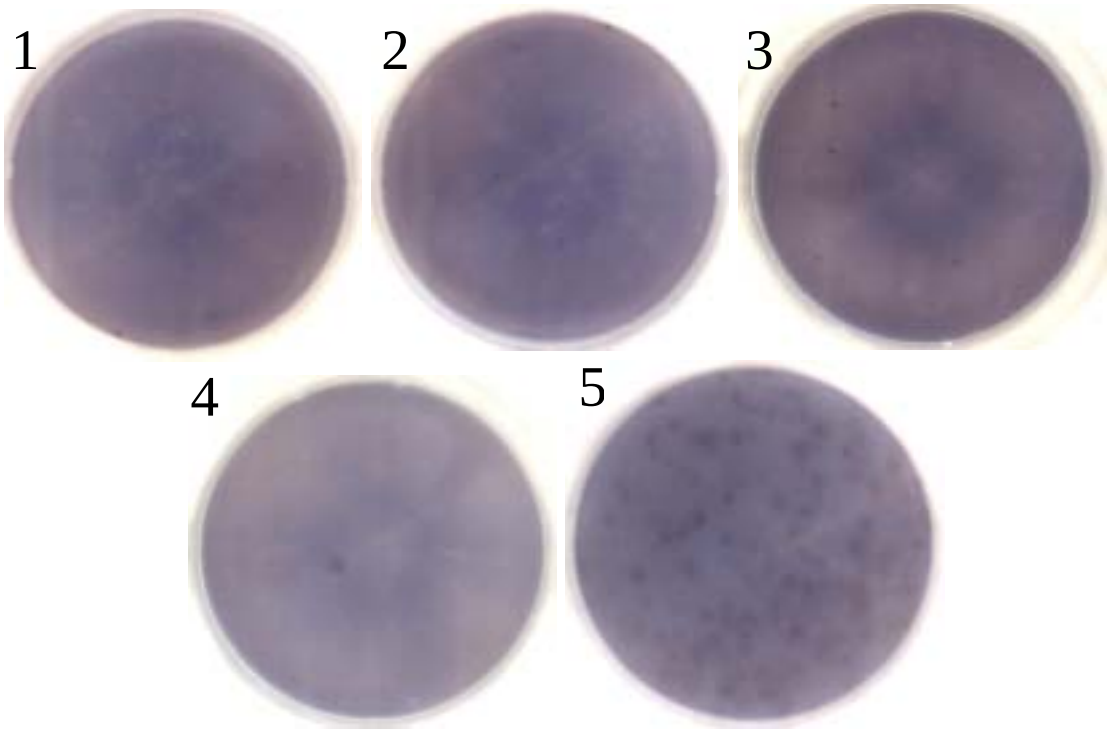

Figure 3

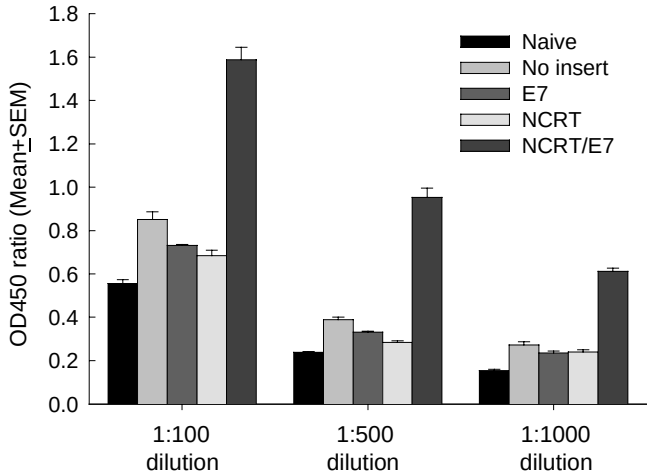

Figure 4

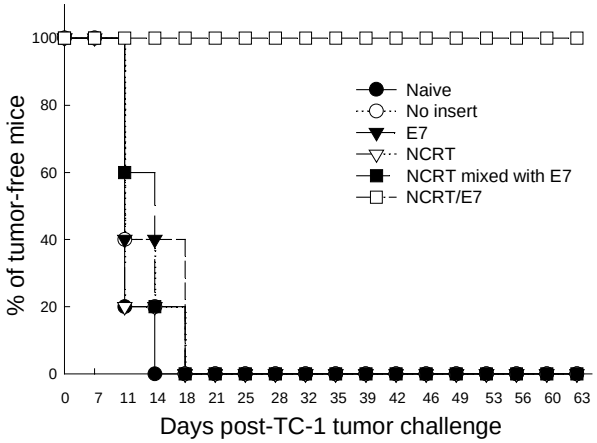

Figure 5