行政院原子能委員會

委託研究計畫研究報告

(轉移性腫瘤動物模型建立及體內放射治療應用研究)

(Establishment of metastatic animal tumor models and their application in internal radiotherapy**)**

計畫編號：1012001INER062

受委託機關(構)：臺北榮民總醫院

計畫主持人：顏上惠

聯絡電話：(02) 2875-7270 ext. 248

E-mail address：shyen@vghtpe.gov.tw

核研所聯絡人員：徐維荃

報告日期：101 年 12 月 12 日

目　錄

中文摘要

核研所發展之脂質體包覆銖-188 藥物(Re^{188}-liposome)已在皮下及肺部轉移之大腸癌動物模型中顯現療效。為探索脂質體包覆銖-188 藥物抗癌作用以發揮這種新治療的最大潛力，我們持續進行改良脂質體包覆銖-188 藥物(Re^{188}-liposome) 的工作。表皮生長因子(EGF)及其受體(EGFR)在癌細胞的增殖，生存，轉移和血管形成扮演非常重要的角色。表皮生長因子及其受體之訊號傳導路徑已被證實為一重要的抗癌藥物靶標。越來越多針對此傳導路徑的靶標治療已通過上市或正在被研究開發中。我們成功製備了上皮細胞生長素標記的EGF-LipoDox，並已證實其對許多上皮細胞生長素受體過度表達的腫瘤細胞有較佳的結合力。此外，我們構建並純化表皮生長因子及酵母菌胞嘧啶脫氨酶之融合蛋白(Fcy-hEGF)以針對表皮生長因子受體過度表達的腫瘤，且轉變 5-氟胞嘧啶(5-fluor°Cytosine)前驅藥物(prodrug)成為具 1000 倍毒性之 5 - 氟尿嘧啶(5-fluorouracil)。我們正嘗試結合 Fcy-hEGF 與脂質體包覆藥物並探討其在上皮細胞生長素受體過度表達的腫瘤之臨床開發應用。

關鍵詞： epidermal growth factor (EGF) 表皮生長因子; epithelial growth factor receptor (EGFR) 表皮生長因子受體; cytosine deaminase 胞嘧啶脫氨酶; fusion protein 融合蛋白; prodrug 前驅藥物; 5-fluor°Cytosine 5-氟胞嘧啶

Abstract

Re^{188}-liposome developed by Institute of Nuclear Energy Research (INER) had shown significant efficacy in subcutaneous and lung metastasis of colorectal cancer in animal models. To demonstrate the greatest potential of this new treatment, we continuously explore the potential indications of Re^{188}-liposome. Human epithelial cancers account for approximately 50% of all cancer deaths. This type of cancer is characterized by excessive activation and expression of the epidermal growth factor receptor (EGFR). The EGFR pathway is critical for cancer cell proliferation, survival, metastasis and angiogenesis. The EGF-EGFR signaling pathway has been validated as an important anticancer drug target. Increasing numbers of targeted therapies against this pathway have been either approved or are currently under development. We are improving Re^{188}-liposome by inserting epidermal growth factor (EGF) to a liposomal drug, LipoDox. The resultant EGF-LipoDox displayed a superior binding affinity for EGF receptor-overexpressing cancer cells. Additionally, We adopted a prodrug system (Fcy-EGF/5-FC) using 5-fluor°Cytosine (5-FC) and human EGF (hEGF) fused with yeast cytosine deaminase (Fcy) to target EGFR-overexpressing cancer and convert 5-FC to a 1000-fold more toxic chemodrug, 5-fluorouracil (5-FU). We will continue exploring incorporate this Fcy-EGF/5-FC prodrug system with liposomal drug for treatment of EGFR-expressing cancers.

Key words: epidermal growth factor (EGF), epidermal growth factor receptor (EGFR), cytosine deaminase, fusion protein, 5-fluor°Cytosine (5-FC)

壹、計畫緣起與目的

Nanoscale liposomes as drug delivery systems containing chemotherapy drugs have been widely used for treatment of cancer [1,2]. As compared with conventional chemotherapy, circulation of liposome coupled chemodrugs could be prolonged. Moreover, the liposome coupled drugs could be redirected to relatively leaky tumor-ass°Ciated blood vessels, leading to superior accumulation in tumors [3,4]. Although liposomal doxorubicin displayed superior l°Calization of doxorubicin in relatively leaky tumor microenvironment, killing of tumor cells required release of this chemodrug and the coupling to its target, DNA. Institute of Nuclear Energy Research (INER) had successfully developed a liposomal therapeutics, 188Re-BMEDA-labelled pegylated liposome (188Re-liposome), and examined its biodistribution, pharmacokinetics and cytotoxic effects, compared with unencapsulated 188Re-BMEDA control in a subcutaneous murine C26-colon tumor model[5]. Series of preclinical efficacy and toxicity studies had shown encouraging results and a phase 0 exploratory human clinical trial has been currently underway with six patients has been injected this therapeutics as of November of 2012.

The goal of this project is to generate an active targeting liposomal drug specific for EGFR-expressing cancer cells. Our previous experiment had demonstrated significant cytotoxic effect of EGF-LipoDox, which is 10-100 folds more potent than LipoDox for killing of EGFR-overexpressing tumor cells, such as A431, BT474, and MDA-468, etc. EGF-LipoDox is internalized into EGFR-overexpressing BT474 to a much greater extent as compared with

LipoDox in the absence of EGF-tag. Based on the preliminary success of EGF-LipoDox, we continue working on cloning, expression and purification of various modified forms of EGF for better coupling of liposomal drug with enhanced affinity to EGFR.

The majority of human epithelial cancers are marked by functional activation of growth factors and receptors of the epidermal growth factor receptor (EGFR) family. Signaling pathways governed by the EGF-EGFR axis play central roles in cancer cell proliferation, survival, metastasis and angiogenesis [6]. Several EGFR antagonists are currently available for the treatment of four metastatic epithelial cancers: non-small-cell lung cancer, squamous-cell carcinoma of the head and neck, colorectal cancer, and pancreatic cancer [7]. These types of cancer account for more than 50% of all cancer deaths.

The overexpression of EGFR on these cancer cells is a well-characterized drug target. Two classes of EGF-EGFR inhibitors, mon°Clonal antibodies and small-molecule tyrosine kinase inhibitors, have been successfully tested and are now in clinical use. Anti-EGFR mon°Clonal antibodies, such as cetuximab (erbitux), bind to the extracellular domain of EGFR and compete for receptor binding by °Ccluding the ligand-binding region, bl°Cking ligand-induced EGFR tyrosine kinase activation. Small-molecule EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib, compete with ATP for the intracellular catalytic domain of the EGFR tyrosine kinase, inhibiting EGFR autophosphorylation and downstream signaling [6]. Although these inhibitors of the EGF-EGFR signaling pathway have achieved significant success in treating variable epithelial cancers, resistance °Ccurs in a significant proportion of patients through various

mechanisms, including mutation of the tyrosine kinase domain, compensation by other oncogenic pathways, etc. [8,9].

To design a novel therapeutic targeting the EGFR signal pathway, we constructed a fusion gene encoding human EGF linked to a yeast cytosine deaminase (CD), Fcy. This approach takes advantage of the enzymatic ability of cytosine deaminase to convert a relatively safe molecule, 5-fluor°Cytosine (5-FC), into a very commonly administered chemotherapeutic, 5-fluorouracil (5-FU), which is 1000-times more toxic than 5-FC. 5-FC has been administered at a dose of 150-200 mg/kg for the treatment of fungal infections with a favorable safety profile [10]. Given that many EGFR-overexpressing cancers, such as head and neck, pancreatic, colon-rectal cancers, etc., were often treated by 5-FU, this EGFR targeting prodrug system could circumvent the high systemic cytotoxicity of 5-FU by concentrating the production of 5-FU at the EGFR-expressing tumor sites. 5-FU is a small molecule capable of diffusing in and out of cells, leading to a significant bystander effect without the requirement of direct cell-to-cell contact.

貳、研究方法與過程

一、 Cloning of DNA in the yeast expression vector.

The DNA sequence encoding Fcy was PCR amplified using a cDNA library that was obtained from yeast as template, whereas human EGF was PCR amplified using a cDNA library derived from a human cancer cell line, SKOV3-ip1. The resulting PCR products were cut with BamHI and EcoRI, which were introduced in PCR primers, and ligated into the protein expression vector, pPICZ-αA, which was cut with the same enzymes. Fcy and hEGF were individually cloned into this vector after the α-secreting signal peptide at the N-terminus, and the C-terminus of the pPICZ-αA vector has a c-myc and hexa-histidine (myc-his_6) tag for convenient protein recognition and purification. Another Fcy PCR product, containing a BamHI cloning site engineered at both the 5’ and 3’ ends, was digested with BamHI and cloned into the pPICZ-αA-hEGF-myc-his_6 vector that was previously cut with BamHI and treated with calf intestinal alkaline phosphatase (CIAP) to prevent self-ligation of the vector.

二、 Expression and protein purification.

Fcy-hEGF-myc-his_6, hEGF-myc-his_6, or Fcy-myc-his_6 were transformed into wild-type X-33 Pichia pastoris, plated on Ze°Cin-containing (200 μg/mL) agar plates and incubated for 3-4 days until the appearance of colonies. Individual colonies were screened for high expression of the individual proteins, as detected using an antibody against c-myc. For large-scale expression, 0.5

liters of BMD medium was in°Culated with the selected colony and grown in shaker flasks to an OD_{600} of 8-10. Protein expression was induced with the daily addition of up to 1% methanol. Three days after induction, the protein-containing culture medium was collected and subjected to filtering before being loaded onto a nickel-resin column (Qiagen, Valencia, CA). The column was washed with 10 column volumes of PBS buffer containing 5 mM imidazole, and the bound proteins were eluted with increasing concentrations of imidazole, using an ÄKTAprime plus purification system (*GE*. Healthcare, Piscataway, NJ). The proteins were characterized on SDS-PAGE gels by staining with Coomassie-blue and Western blot analysis using an antibody specific for c-myc.

三、 **Preparation of EGF-LipoDox**.

The pr°Cess adopted for generation of EGF-LipoDox was previously described by Chen etl al. [11]. For the synthesis of hEGF-his_6-PEG–DSPE molecule, hEGF-his_6 was dissolved in PBS–EDTA and mixed at 1:1.2 molar ratio with N-succinimidyl 3-(2-pyridyldithio) propionate dissolved in DMSO [12]. After 1 h in room temperature, the mixture will be lyophilized, and dissolved in the solution containing tris(2-carboxyethyl) phosphine under nitrogen to expose the –SH group. The thiolated protein or peptide will be then added to the MAL–PEG2000–DSPE micelle solution at 5:1 molar ratio while maintaining mixing under nitrogen at 10 °C overnight. HPLC analysis will be used to confirm that most of MAL–PEG–DSPE molecules were conjugated with the hEGF-his6 after such reactions. Ligand-conjugated lipids will be transferred into preformed 188Re-Dox-liposome based on the pr°Cedure

developed by Ishida et al. [13] with minor modifications. hEGF-his6–PEG–DSPE solution will be added into the preformed liposome solution at 9:100 molar ratio and incubated at 50 °C for 30 min. The solutions will be then dialyzed against PBS for 4 h to remove unconjugated hEGF-his6. Non-targeted liposomes were prepared similarly by substituting hEGF-his_6-conjugated lipid with mPEG2000–DSPE.

四、 **Detection of doxorubicin uptake by cancer cells**.

EGFR-overexpressing MDA-MB-468 and EGFR low expresser, MDA-MB-231, cancer cells were plated in 6-well plate ($5x10^5$ cells/well) one day before experiment. The cells were incubated with 10mM of either LipoDox or EGF-LipoDox at 37 °C for 30 minutes. The cells were later washed 3 times with PBS followed by lysis. The amounts of doxorubicin were detected and quantified using the intrinsic fluorescence of doxorubicin (excitation wavelength, 480 nm; emission wavelength, 580nm.

五、 **In vitro binding of Fcy-hEGF, hEGF, and Fcy to purified EGFR.**

Purified EGFR (*R&D Systems*, Minneapolis, MN) was diluted in coating buffer (0.2 M sodium carbonate/bicarbonate pH 9.4, 0.5 μg/ml) and immobilized on an ELISA plate by incubation at 4°C overnight. Various concentrations of Fcy-hEGF, hEGF, and Fcy (0-50 nM) were incubated with immobilized EGFR at room temperature for one hour, followed by washing the ELISA plate three times with PBS buffer. The in vitro binding of each his_6-tagged proteins with EGFR was detected using an HRP-tagged,

anti-his_6 antibody and developed by the addition of the HRP substrate (100 μl/well), 3,3',5,5'-tetramethylbenzidine (TMB). The peroxidase reaction was stopped 30 min after the addition of 0.5 M H_2SO_4 (50 μl/well), and the absorbance was measured at 450 nm with a multichannel microtiter plate reader.

六、 In vitro enzymatic activity of Fcy-hEGF and Fcy.

The enzymatic activities of Fcy - hEGF and Fcy were determined by measuring the production of 5-FU in the presence of 5-FC. Ten picomoles of either Fcy-hEGF (0.28 μg) or Fcy (0.20 μg) was mixed with increasing concentrations of 5-FC (0, 0.03, 0.1, 0.3, 1, and 3 mM) in 1 ml PBS buffer to initiate the conversion of 5-FC to 5-FU at room temperature. Two microliters of the reaction was collected every three minutes and the fluorescent intensities of 5-FC and 5-FU were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The absorbance at 255 and 290 nm was used to calculate the concentrations of 5-FU and 5-FC, using a formula deduced by Senter et al., [14]: [5-FU] mM = 20 x (0.185 x A255 - 0.049 x A290); [5-FC] mM = 20 x (0.119 x A290 - 0.025 × A255). The rates of 5-FU production and 5-FC depletion under various conditions of either Fcy-hEGF or Fcy admixed with increasing concentrations of 5-FC were used to calculate the V_{max} and K_m.

七、 Fcy-hEGF, hEGF, and Fcy binding to EGFR-expressing cells.

The expression level of EGFR in A431, MDA-MB-468, MDA-MB-231, and MCF-7 cells was analyzed with Fluorescence

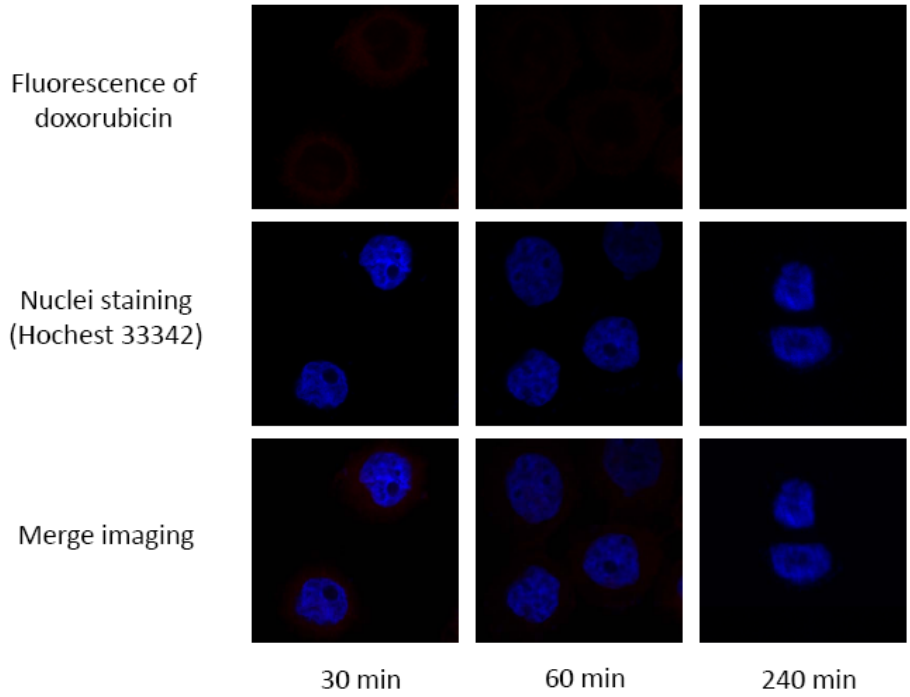
Activated Cell Sorting (FACS). Cell surface EGFR was detected with an anti-EGFR antibody, cetuximab (erbitux), which was subsequently bound by a FITC-tagged goat anti-human IgG antibody. To demonstrate the binding ability of purified Fcy-hEGF, hEGF, and Fcy to cell surface EGFR, A431, MCF-7, MDA-MB-468, and MDA-MB-231 cells were incubated with the indicated his6-tagged protein for 1 hour. A FITC-labeled antibody specific for his6-tag was later incubated with the cells, which were subjected to FACS analysis. The fluorescent intensities represent the amount of EGFR detected by erbitux or his_6-tagged proteins on each cancer cell line.

八、 MTT assays for the measurement of cell viability.

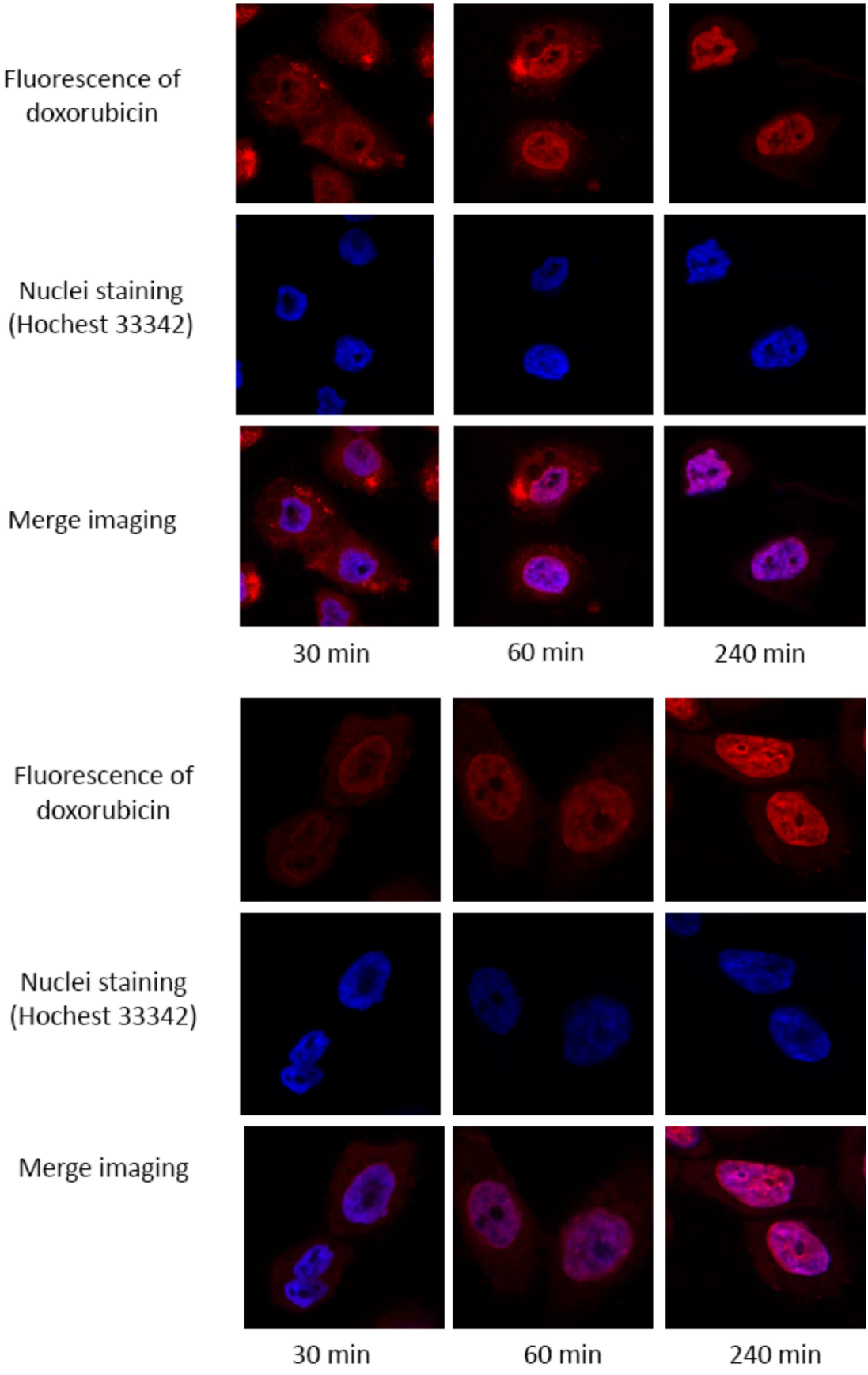
A431, MDA-MB-468, MDA-MB-231 and MCF-7 (5000 cells/well) cells were incubated with combinations containing different concentrations of 5-FC and Fcy-hEGF, Fcy, or hEGF. Two groups were tested at different incubation times with the proteins. One group was treated continuously with both 5-FC and the indicated proteins, whereas the proteins were removed from the other group after one hour of incubation by washing with PBS three times before the addition of 5-FC. Cells were plated in a 96-well plate and subjected to the MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) assay three days after the addition of the indicated proteins and 5-FC. Twenty-five microliters of MTT solution (5 mg/ml in PBS) was added to the cells. After a 2-h incubation, MTT was removed and the cells were washed with PBS, followed by addition of 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide). After a 4-hour

incubation at 37°C, the optical densities were measured at 570 nm, and the extraction buffer alone served as a blank.

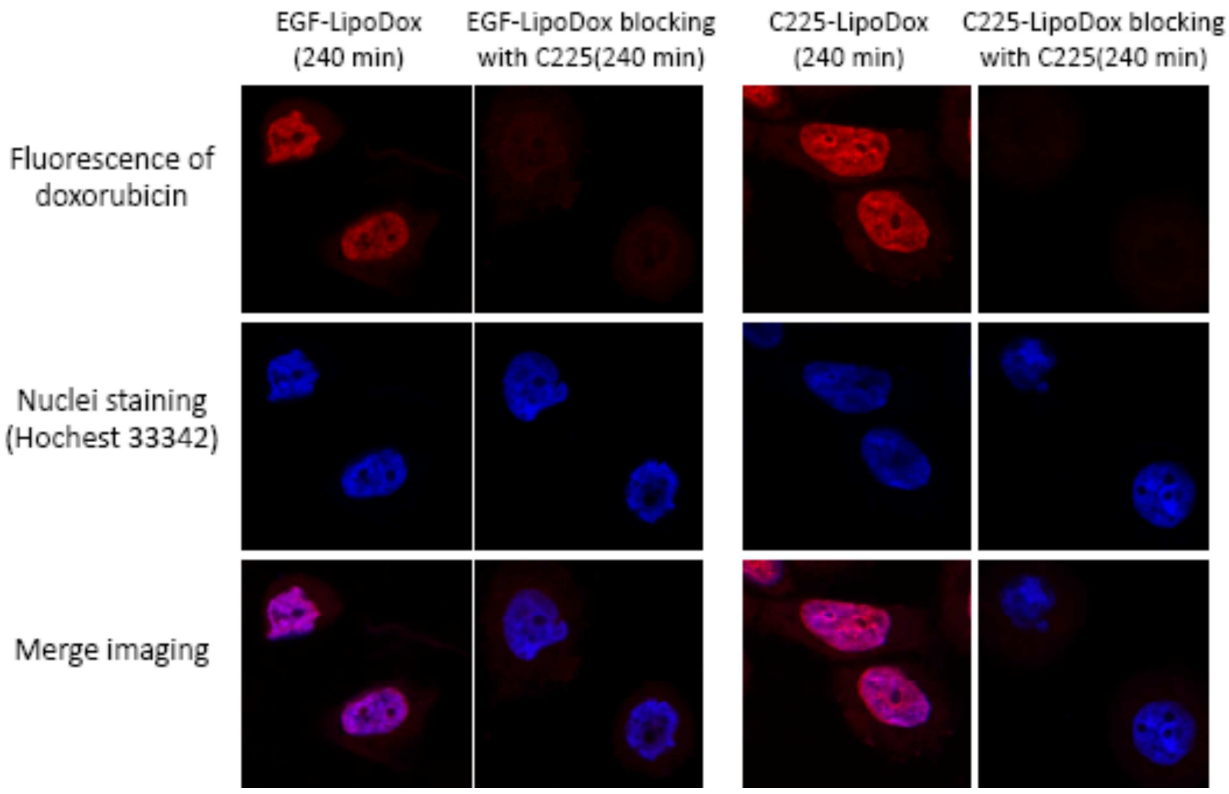
參、主要發現與結論



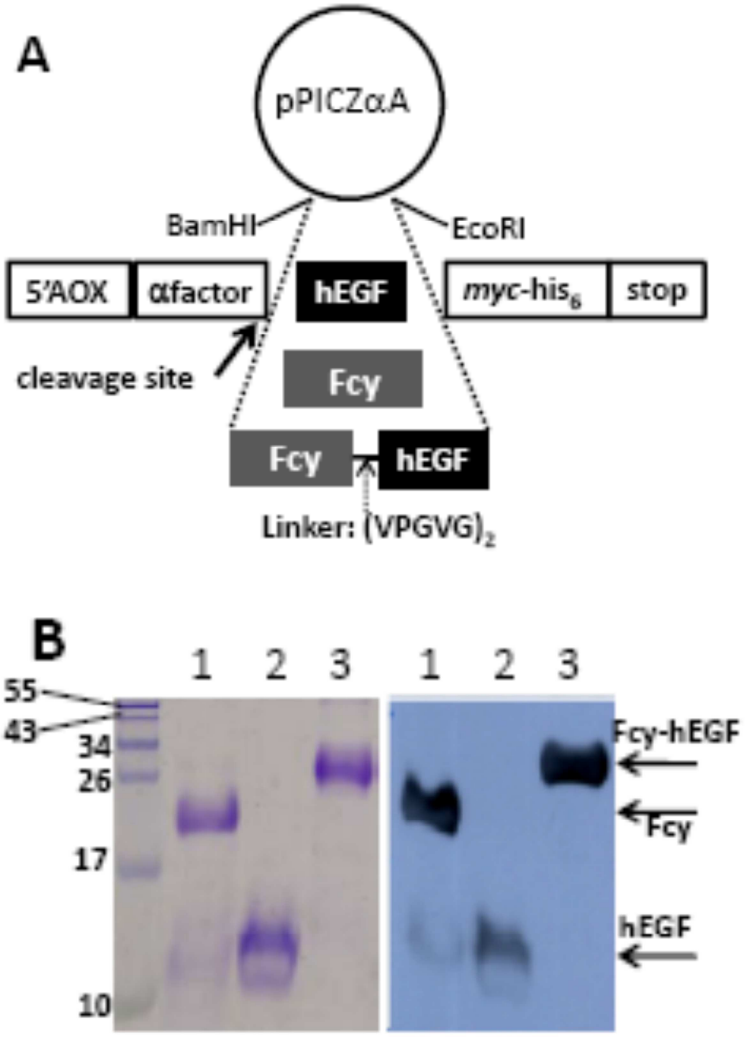
(Fig. 1) Imaging of LipoDox distribution in MDA-MB468 cells (by Conf°Cal Fluorescence Microscopy). In the absence of tumor targeting moiety, there is no significant amount of fluorescence contributed by doxorubicin.


Fluorescence of doxorubicin
Nuclei staining (Hochest 33342)
Merge imaging
30 min
60 min
240 min
Fluorescence of doxorubicin
Nuclei staining (Hochest 33342)
Merge imaging
30 min
60 min
240 min

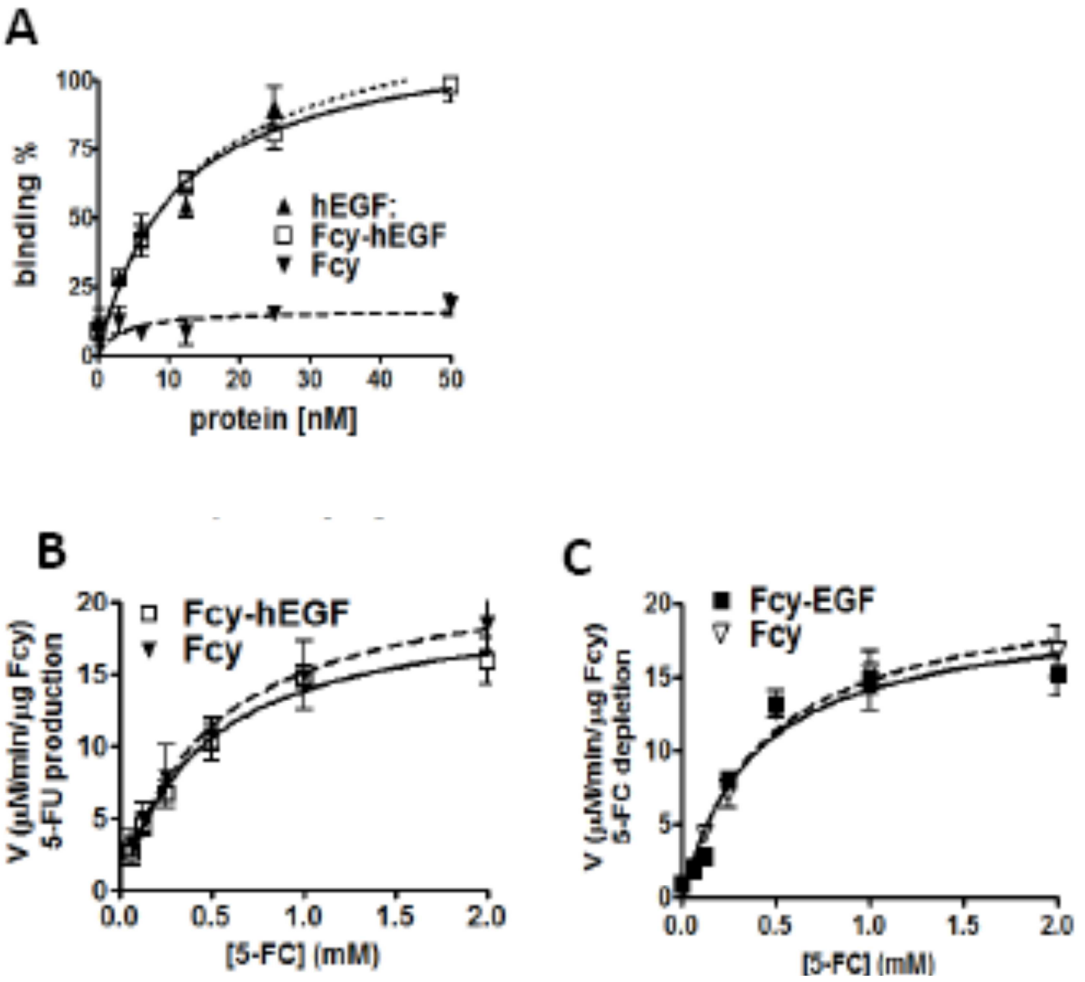
(Fig. 2) Imaging of C225-LipoDox (right panel) and EGF-LipoDox (left panel) distribution in MDA-MB468 cells (by Conf°Cal Fluorescence Microscopy). These two EGFR-targeting LipoDox displayed significant increase of fluorescence resulted from doxorubicin.



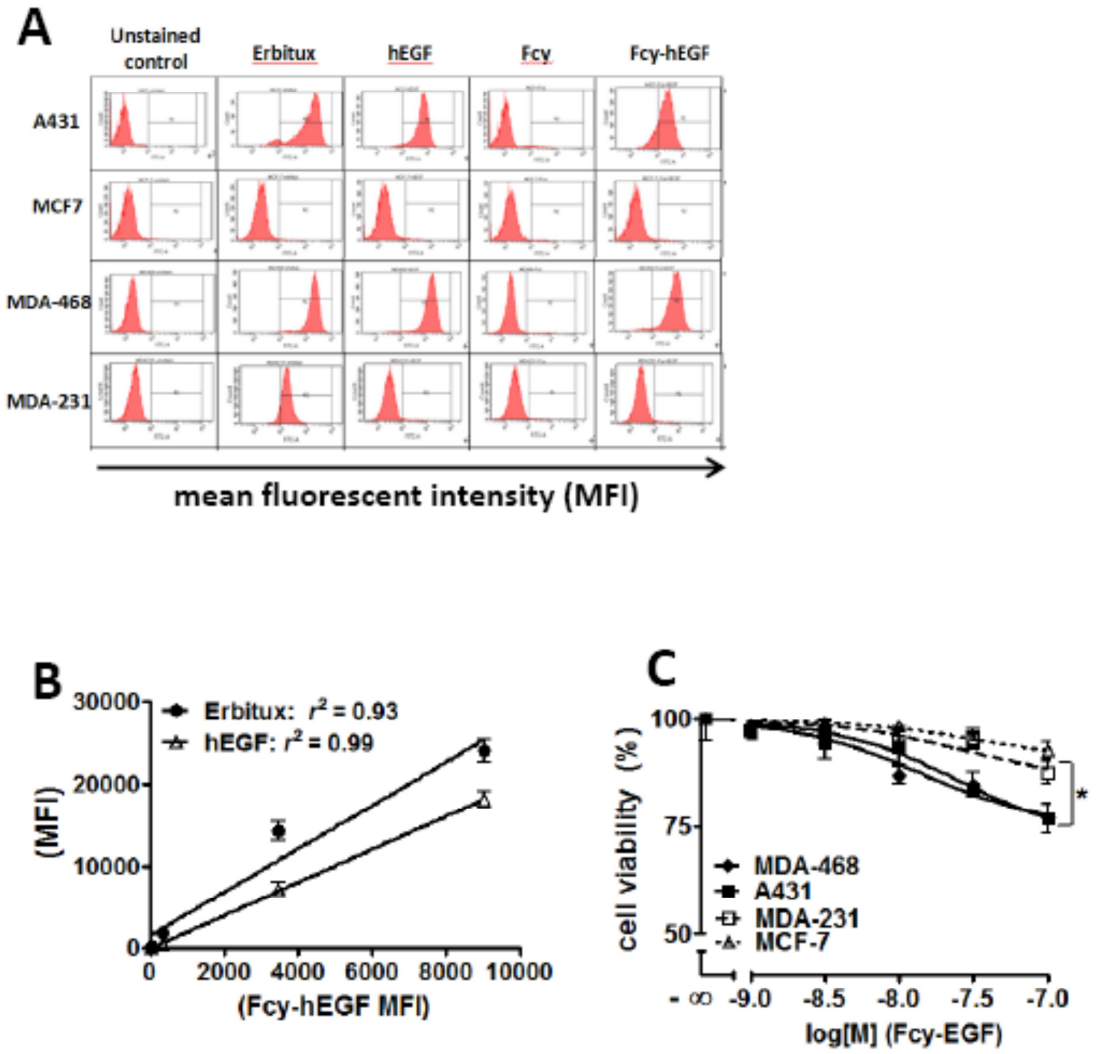
(Fig. 3) C225 bl°Cks doxorubicin fluorescence uptake by MDA-MB468 cells treated with EGF-LipoDox and C225-Lipodox.



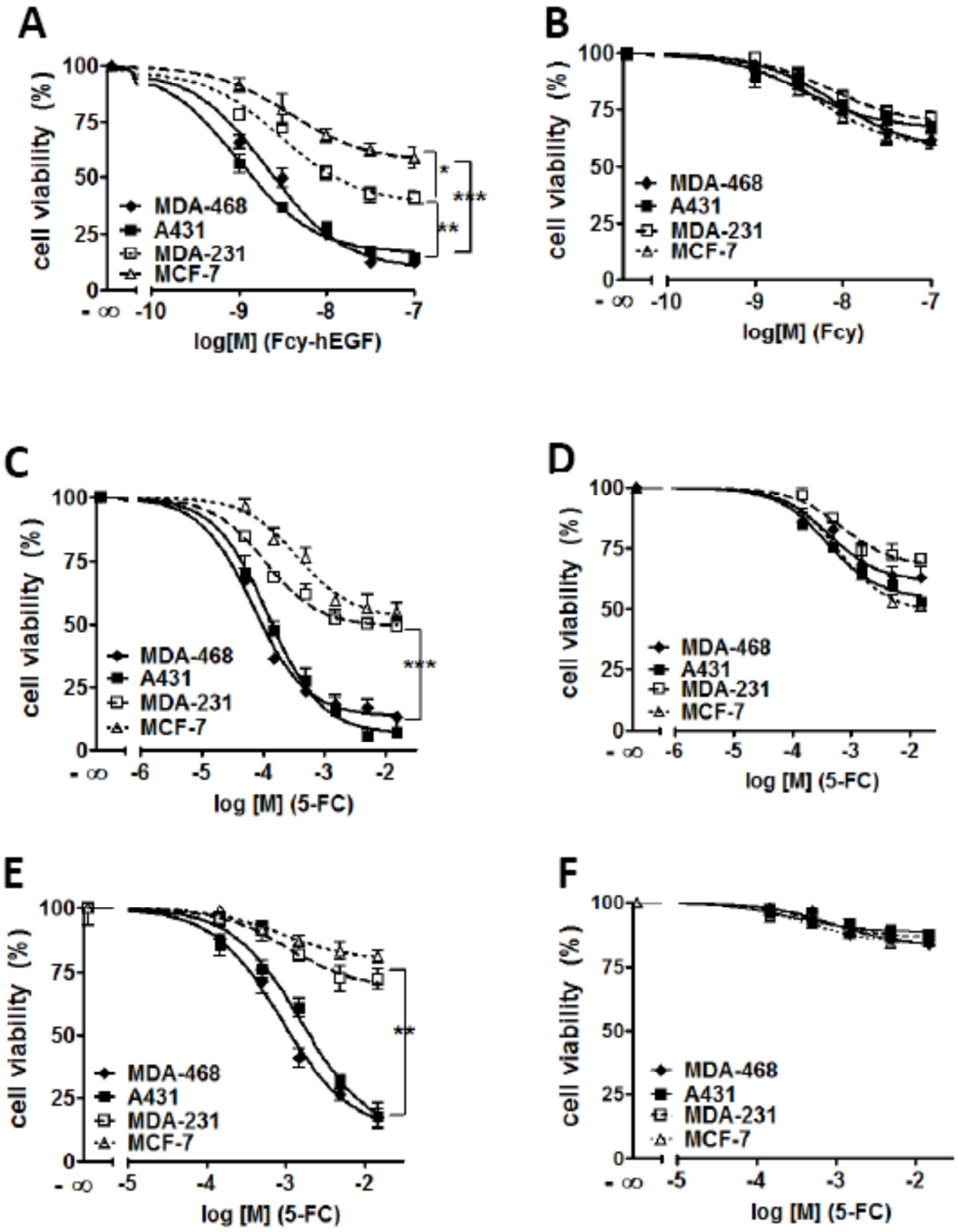
(Fig. 4) Schematic diagram and purification of proteins. (A) The genes encoding Fcy-hEGF, hEGF-myc-his6, and Fcy were cloned into the yeast vector, pPCIZ**α**A, and digested with the restriction enzymes, BamHI and EcoRI. (B) Coomassie staining (left panel) and Western blot analysis (right panel) of purified Fcy (lane 1), hEGF (lane 2) and Fcy-hEGF (lane 3) proteins.



(Fig. 5) Bioactivities of Fcy-hEGF. (A) In vitro binding of Fcy-hEGF, hEGF and Fcy to purified EGFR immobilized on an ELISA plate was examined using an HRP-tagged anti-his_6 antibody. The binding affinities of Fcy-hEGF and hEGF for EGFR are 11 ± 2 and 13 ± 3 nM, respectively, whereas no discernible coupling between Fcy and EGFR was noted. (B) The rates of 5-FU production by 10 pmole of Fcy-hEGF (0.28 μg) and Fcy (0.20 μg) were determined in the presence of increasing concentrations of 5-FC (0-3 mM). The 5-FU production rates were plotted against the concentrations of 5-FC. Each experiment was repeated in duplicate, in at least three experiments.



(Fig. 6) Fcy-hEGF, hEGF, and Fcy bind to cells with various EGFR expression levels. (A) The expression level of EGFR was analyzed in A431, MDA-MB-468, MDA-MB-231 and MCF-7 cells with FACS using an anti-EGFR antibody, erbitux. The binding of Fcy-hEGF, hEGF, and Fcy to cell surface EGFR was determined using an anti-his_6-tag antibody labeled with FITC. (B) The mean fluorescent intensity (MFI) obtained from the binding of erbitux and hEGF was plotted against that of Fcy-hEGF binding to individual cell lines. (C) Fcy-hEGF alone exhibits suppressive effects on EGFR-overexpressing MDA-468 and A431 cells compared with MDA-231 and MCF-7 cells (asterisk indicates $p<0.05$).



(Fig. 7) Fcy-hEGF combined with 5-FC displays a significant inhibitory effect on EGF-overexpressing MDA-468 and A431 cells. (A) In the presence of 5-FC (1 mg/ml), we observed marked dose-dependent inhibition by Fcy-hEGF in MDA-468 and A431 cells, with IC_{50} values of 2.1 and 1.0 nM, respectively. In contrast, the inhibitory effect of

Fcy-hEGF on MDA-231 and MCF-7 plateaued at 40% and 58% cell viability. (p value of MDA-468/A431 vs. MDA-231 and MCF-7: <0.05 and <0.001, respectively). (B) No discernible difference was detected with increasing concentrations of Fcy on the cell lines tested. (C&D) Dose-response curves of 5-FC were obtained by continuously treating cells with either Fcy-hEGF (C) or Fcy (D). As opposed to the Fcy-treated group, significant differences were observed between cell lines that expressed high (MDA-468 & A431) and low (MDA-231 & MCF-7) levels of EGFR ($p<0.005$ in Fcy-hEGF treated group; triple asterisks). (E&F) Similar experiments were conducted as in Fig 4 C&D, except the cells were incubated with either Fcy-hEGF (E) or Fcy (F) for only one hour before washing with PBS and the addition of 5-FC. In the Fcy-hEGF treated group, 5-FC treatment resulted in a more significant suppressive effect on both MDA-468 and A431 cells compared with MDA-231 and MCF-7 cells ($p<0.005$, triple asterisk).

肆、參考文獻

[1] M. Ferrari, Frontiers in cancer nanomedicine: directing mass transport through biological barriers, Trends Biotechnol 28 (2010) 181-188.

[2] R. Singh, J.W. Lillard, Jr., Nanoparticle-based targeted drug delivery, Exp Mol Pathol 86 (2009) 215-223.

[3] D.C. Drummond, C.O. Noble, M.E. Hayes, J.W. Park, D.B. Kirpotin, Pharmacokinetics and in vivo drug release rates in liposomal nan°Carrier development, J Pharm Sci 97 (2008) 4696-4740.

[4] H. Maeda, G.Y. Bharate, J. Daruwalla, Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect, Eur J Pharm Biopharm (2008).

[5] Y.J. Chang, C.H. Chang, T.J. Chang, C.Y. Yu, L.C. Chen, M.L. Jan, T.Y. Luo, T.W. Lee, G. Ting, Biodistribution, pharmacokinetics and microSPECT/CT imaging of 188Re-bMEDA-liposome in a C26 murine colon carcinoma solid tumor animal model, Anticancer Res 27 (2007) 2217-2225.

[6] F. Ciardiello, G. Tortora, EGFR antagonists in cancer treatment, N Engl J Med 358 (2008) 1160-1174.

[7] I. Vivanco, I.K. Mellinghoff, Epidermal growth factor receptor inhibitors in oncology, Curr Opin Oncol 22 (2010) 573-578.

[8] C. Ma, S. Wei, Y. Song, T790M and acquired resistance of EGFR TKI: a literature review of clinical reports, J Thorac Dis 3 (2011) 10-18.

[9] G. Giaccone, Y. Wang, Strategies for overcoming resistance to EGFR family tyrosine kinase inhibitors, Cancer Treat Rev 37 (2011) 456-464.

[10] A. Vermes, H.J. Guchelaar, J. Dankert, Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions, J Antimicrob Chemother 46 (2000) 171-179.

[11] L.C. Chen, C.H. Chang, C.Y. Yu, Y.J. Chang, Y.H. Wu, W.C. Lee, C.H. Yeh, T.W. Lee, G. Ting, Pharmacokinetics, micro-SPECT/CT imaging and therapeutic efficacy of (188)Re-DXR-liposome in C26 colon carcinoma ascites mice model, Nucl Med Biol 35 (2008) 883-893.

[12] S. Song, D. Liu, J. Peng, Y. Sun, Z. Li, J.R. Gu, Y. Xu, Peptide ligand-mediated liposome distribution and targeting to EGFR expressing tumor in vivo, Int J Pharm 363 (2008) 155-161.

[13] T. Ishida, D.L. Iden, T.M. Allen, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, FEBS Lett 460 (1999) 129-133.

[14] P.D. Senter, P.C. Su, T. Katsuragi, T. Sakai, W.L. Cosand, I. Hellstrom, K.E. Hellstrom, Generation of 5-fluorouracil from 5-fluor°Cytosine by mon°Clonal antibody-cytosine deaminase conjugates, Bi°Conjug Chem 2 (1991) 447-451.