Abstract. The E gene from ΦX174 encodes a membrane protein with a toxic domain that leads to a decrease in the tumour cell growth rate. With the aim of improving the antitumour effect on lung and colon cancer cells of the currently used chemotherapeutic drugs such as gemcitabine, carboplatin and paclitaxel, and 5-fluorouracil (5FU) plus folinic acid (FA) with irinotecan or oxaliplatine, we investigated a new combined therapy using these drugs associated to the transfection of E gene. Our results showed that E gene was able to decrease proliferation rate in A-549 and T-84 cells by inducing apoptotic the mitochondrial pathway. Significantly greater inhibition of proliferation was obtained using drugs in combination with E gene in comparison to single-agent treatments or controls. E gene combined with paclitaxel had the greatest effect on A-549 cells and combined with 5FU/FA/oxaliplatin on T-84 cells. Antitumour mechanisms of the chemotherapeutic drugs were enhanced by E gene, which itself has direct oncolytic effects inducing A-549 and T-84 apoptosis. Our in vitro results indicate that the combined therapy of E gene and cytotoxic drugs may be of potential therapeutic value as a new strategy for patients with advanced lung and colon cancer.

Introduction

Lung and colorectal cancer are two of the most frequent tumours in the developed world. During the past few years, several novel drugs have improved the clinical response of both tumour types. Paclitaxel (Pac), docetaxel (Doc), gemcitabine (Gem) and vinorelbine have shown significant activity against non-small cell lung cancer (NSCLC) which represents ~5-80% of all lung cancers (1,2). On the other hand, new chemotherapeutic agents, such as oxaliplatin (Oxa), irinotecan (CPT-11) and capecitabine and some biologic agents have contributed considerably to the treatment of colon cancer (3). In fact, 5FU and folinic acid (FA)-based combination regimens that include Oxa and CPT-11 have shown an increase in the survival in patients with metastatic disease (4,5). However, in spite of these advances, the majority of patients diagnosed of either NSCLC or colon cancer have an advanced stage of disease and a poor prognosis. A major goal of lung and colon cancer research is to improve the effectiveness of these cytotoxic agents so the development of new therapeutic strategies is necessary.

To enhance the efficacy of antitumour drugs in patients with advanced cancer is one of the main objectives of the new gene therapy systems. Positive results of these strategies have been reported in bladder, pancreas and breast cancer (6-8). In lung cancer, the telomerase-specific oncolytic adenovirus combined with Gem resulted in a synergistic cytotoxic effect (9). Adeno-associated virus (AAV) vector encoding human CD40L, which is able to activate host immune systems, has been associated with Car in lung cancer cells (A-549 cell line). The results showed an increase in the antitumor response suggesting that this combined therapy may provide an effective form of lung cancer treatment (10). Recently, we have demonstrated that the use of Pac in the same cell line associated to gef gene transfection, a gene with cell-killing function, enhanced the chemotherapeutic effect of this drug (11). Colon cancer has demonstrated to have efficacy of gene therapy cytotoxic-combined strategies. Using colon cancer cell lines (HT-29, WiDr and HCT-116) the antitumoural activity of NV1020, an oncolytic herpes simplex virus type 1, was increased by 5FU, SN38 or Oxa (12). On the other hand, RNA interference has been used to down-regulate the NF-κB p65 subunit increasing sensitivity to chemotherapeutic agents in the HCT116 colon cancer cell line. In fact, in vivo administration of p65 siRNA reduced...
Cells were seeded into 24-well plates (10⁴ cells per well). Transfection. One day before transfection, A-549 and T-84 cells were grown in monolayer culture at 37˚C in an atmosphere containing 5% CO₂. The transfection of E gene in melanoma cells induced apoptosis obtaining a significant decrease in the percentage of surviving cells and in the volume of tumour induced in vivo.

The aim of this study is to investigate whether gene therapy with E gene is able to increase the therapeutic effect of drugs used in the classical treatment of lung and colon cancer. Our results clearly demonstrate that E gene significantly enhanced the anti-proliferative activity of Pac in A-549 human lung cancer cells and 5FU-FA and Oxa in T-84 human colon cancer cells. This significant reduction of growth rate in both A-549 and T-84 cell lines was mediated by an apoptotic phenomenon. These results suggest that the therapeutic association of E gene with drug chemotherapy may be a promising therapy for treating patients with advanced lung or colorectal cancer.

Materials and methods

Cell culture. The lung carcinoma cell line A-549 (ATCC-CCL185) and the colon carcinoma cell line T-84 (ATCC CCL-248) were grown with DEMEM (Sigma Chemical Co., St. Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 40 mg/l gentamycin and 500 mg/l ampicillin (Antibióticos S.A, Spain). Cells were maintained in monolayer culture at 37˚C in an atmosphere containing 5% CO₂.

Vector construction. The E gene (provided by Dr J.L. Ramos from the Zaidín Experimental Station, CSIC, Granada, Spain) was amplified using specific primers (sense 5’-CATGGTT AGGCTGGACTTTGT-3’ and antisense 5’-TCCTTCTGCGAAGTGTC-3’). The resulting plasmid pCDNA3.1 vector following the manufacturer’s instructions (Invitrogen). The plasmid DNA was amplified by using specific primers (sense 5’-ATCATGGT TTGAGACCTTCAA-3’ and antisense 5’-CATCTCTTGCTGGAAGTTCA-3’). Images were scanned and analysed using a Bio-Rad documentation system (Quantity One Analysis Software).

Microscopic analysis. E/V5 fusion protein expression was confirmed in A-549- and T-84 pcDNA3.1/E-transfected cells using an anti-V5-FITC antibody (Invitrogen). Briefly, the cells were grown on coverslips, washed with PBS, fixed in 100% methanol (room temperature) for 5 min, blocked with 1% bovine serum albumin/PBS, and then incubated with anti-V5-FITC antibody diluted (1:500) in 1% bovine serum albumin/PBS for 1 h. DAPI (Invitrogen) solution (100 nM) was used for nuclear staining. The cells were then rinsed briefly with PBS, mounted and visualized using fluorescent microscopy (Hitachi H7000 transmission electron microscope) was used, according to Prados et al (11) to determine morphological changes in parental and transfected A-549 and T-84 cells.

In vitro cell proliferation assay. Cells were seeded in 24-well plates (10⁴ cells per well), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/ml) was added to each well (20 μl) and incubated 4 h at 37˚C. Then, 200 μl of dimethylsulfoxide (DMSO) was added to each well after the medium was removed. Optical density was determined using a Titertek multiscan colorimeter (Flow, Irvine, CA) at 570 and 690 nm. Cells transfected with empty vector were used as controls. The proliferation effect of the combined gene and cytotoxic therapy was determined for 7 days using Pac, Gem and Car (all drugs to 100, 50 and 1 μM) for A-549 cells (18,19) and 5FU (500, 500 and 5 μM), CPT-11 (200, 50 and 10 μM), Oxa (100, 10 and 0.5 μM) and FA (5 μM) for T-84 cells (20,21).

Mitochondrial transmembrane potential and apoptosis analysis. Changes in mitochondrial transmembrane potential were evaluated by labelling parental and transfected A-549 and T-84 cells with DiOC₆ (40 nM) for 15 min at 37˚C. Cells and T-84 cells (70% confluence) were transfected with a final volume of 20 μl of transfection mixture in 500 μl of culture medium per well. Experiments were performed in two groups: A-549 and T-84 cells transfected at day 1 alone (A-549/TE’ and T-84/TE’) and A-549 and T-84 cells transfected at days 1, 3 and 5 (A-549/TE’ and T-84/TE’). A-549 and T-84 cells transfected with empty vector were used as controls.

Reverse transcription-PCR (RT-PCR). Upregulation of mRNA expression of E cDNA was determined by RT-PCR. Total RNA was extracted from transfected and parental cells with the Rneasy mini kit (Qiagen), and cDNA was generated by means of the Promega reverse transcription system using total cellular RNA (1 μg). PCR amplification of gene E took place under the above-described conditions and was run on a 1.8% agarose gel and visualized by ethidium bromide staining. RNA integrity was assessed by amplification of β-actin mRNA (sense 5’-ATCATGGTTTGTACGACCTTCAA-3’ and antisense 5’-CATCTCTTGCTGGAAGTGTC-3’).
were then washed twice with PBS and analyzed directly on a flow cytometer using excitation and emission wavelengths of 495 and 525 nm, respectively. Apoptosis was determined by FACScan flow cytometer using Annexin V-FITC apoptosis detection kit I (BD Pharmingen, San Diego, CA, USA).

**Cellular fractionation and Western blot.** Cells were harvested and lysed in sample buffer consisting of 62.5 mM Tris HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.5% bromophenol blue and 100 mM dithiothreitol. Proteins were separated on 10% SDS-polyacrylamide gels and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The antibodies used in this study were rabbit polyclonal IgG anti-caspase-3, anti-caspase-9 and anti-caspase-8 (Santa Cruz Biotech, Santa Cruz, CA) and anti-β-actin (Sigma Chemical Co.). Blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h and then incubated with the rabbit polyclonal primary antibody (1:1000 dilution) (Santa Cruz Biotech) overnight at 4°C. After addition of peroxidase-conjugated secondary antibody, proteins were added with ECL Western blotting detection reagents (Amersham Biosciences, USA) and analyzed using the Fuji LAS-3000 imaging system (Japan). In order to analyze the redistribution of cytochrome c during apoptosis, cells were fractionated into cytosolic and membrane-bound fractions. For this purpose, cells were exposed to 0.05% digitonin (Sigma) in isotonic sucrose buffer (250 mM sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM ethylenediaminetetraacetate, and 1 EGTA; pH 7.1) for 2 min at room temperature to collect the soluble fraction as cytosolic extracts. Digitonin insoluble fraction was dissolved in 2% sodium dodecyl sulfate (SDS) buffer to collect the membrane-bound part. Immunoblotting to detect cytochrome c was realized as described above using anti-cytochrome c and anti-Bcl-2 (Santa Cruz Biotech) to demonstrate the cellular fractionation.
Immunohistochemical staining. Cells were grown on slides, fixed in 100% methanol (room temperature) for 5 min and stained with haematoxylin-eosin. Immunohistochemistry was performed using an avidin-biotin-peroxidase complex (ABC) detection kit (Dako ChemMate Detection Kit). Endogenous peroxidase activity was inhibited with 15 min incubation in 3% H2O2 peroxidase in methanol. Non-specific antigenic sites were blocked by a 20 min step in diluted normal serum (Dako) before the sections were incubated for 1 h at 37˚C with the primary antibody (rabbit polyclonal IgG anti-caspase-3, anti-caspase-9 and anti-caspase-8 (Santa Cruz Biotech). Then, the sections were first incubated at RT with a biotinylated secondary antibody (Dako) for 30 min and second with the peroxidase-conjugated biotin-avidin complex (ABC) for 40 min. Finally, the peroxidase was revealed by immersion in 3,3′-diaminobenzidine-tetrahydrochloride (DAB, Dako). The section DAB colorization was inspected under the light microscope. Immunostaining of negative control included sequential elimination of the primary or secondary antibody from the staining series.

Statistical analysis. SPSS 7.5 software (SPSS, Chicago, IL) was used for all statistical analyses. Results were compared by using the Student’s t-test. All data are expressed as means ± SD. Differences were considered statistically significant at P<0.05.

Results

E gene expression and E protein localization in transfected A-549 and T-84 cells. The antitumour effect of E gene was analysed by transfecting A-549 and T-84 cells with the pcDNA3.1/E vector. E gene expression in these cells was assessed by RT-PCR. As shown in Fig. 1A, an amplification fragment of 154 bp was found in A-549/TE* and T84/TE* cells, indicating the effectiveness of the construction and...
allowing its use in the subsequent *in vitro* experiment. The expression of the E-V5 fusion protein showed a clear signal in the T-84/TE* cell cytoplasm with a dotted fluorescence pattern at 3 day (Fig. 1B). Similar results were found in A-549/TE* (data no shown).

**E gene induced morphological changes in A-549 and T-84 cells.** Both A-549 and T-84 cells were attached to the bottom of the flasks with an irregular arrangement in confluent cultures (Fig. 2A). Transfected cells with empty vector showed no morphological changes with respect to the parental cell line (data not shown). However, significant differences were observed between control and E gene-transfected cells which showed a progressive loss of monolayer culture uniformity over four days, with the presence of irregular zones without cells. These zones were more evident when the transfection was continuous (Fig. 2A).

**E gene inhibits growth in transfected A-549 and T-84 cells.** As shown in Fig. 2B, the growth of A-549 and T-84 cells transfected with the empty vector was similar to that of the parental cells. In contrast, both tumoral cell lines transfected with E gene showed a significant and time-dependent decrease in growth. Slight differences were found in A-549/TE* and T-84/TE* at the day 2, 5 and 7 (<10%) after transfection with E gene versus empty vector transfected cultures. However, analysis of the A-549/TE** and T-84/TE** cells showed a significant modulation of growth. The determination of % of surviving cells showed an 11.3±0.6% and 11.6±1.1% (day 5) and 19.8±1.3% and 19.2±1.7% (day 7) decrease to A-549 and T-84 cells, respectively (p<0.05) (Fig. 2B).

**E gene induced functional and morphological mitochondrial damage.** Mitochondrial damage was assessed biochemically by measuring DiOC₆. At 48 h after transfection A-549/TE*
and T-84/TE* cells showed a significant decrease in mitochondrial transmembrane potential \((p<0.05)\), thus indicating an increase in mitochondrial membrane permeability after \(E\) gene treatment (Fig. 3A). Moreover, morphological changes of mitochondria were observed by TEM analysis. The most relevant feature found in A-549- and T-84-transfected cells was the presence of dilated mitochondria with clear matrices and disrupted cristae. In contrast, control cells showed unaltered mitochondria (Fig. 3B).

**E** gene induces caspase protein expression. Immunohistochemical analysis showed caspase-3- and -9-positive staining of A-549- and T-84-transfected cells. In contrast, caspase-8 study was negative in both transfected cells (Fig. 4A). Western blot analysis showed presence of cleaved caspase-9 and -3 in \(E\) gene-transfected cells (Fig. 4B) and a cytochrome c release to cytoplasm (Fig. 4C). These findings strongly suggest that \(E\) gene therapy system may upregulate caspase-3 and -9 expression in the transfected tumour cells and promotes apoptosis by mitochondrial pathway.

Combined \(E\) gene-cytotoxic therapy induced growth disturbance on A-549 and T-84 cell. After establishing the effect of \(E\) gene on the lung and colorectal cancer cells growth, we investigated its use in a combined therapy with conventional cytotoxic drugs. Non-transfected A-549 cells treated with Pacl, Car or Gem and non-transfected T-84 cells treated with 5FU/FA/Oxa or 5FU/FA/CPT-11 showed a significant reduction in cell viability. A-549/TE*, A-549/TE**, T-84/TE* and T-84/TE** cells showed a greater rate of proliferation inhibition with the combined therapy than was observed with the drugs or \(E\) gene alone. Fig. 5 shows that Gem induced a significant growth decrease in A-549 cells. This proliferation inhibition was time- and concentration-dependent, finding the greatest inhibition at day 7 and at the highest concentration \((36.9\pm2.6\%)\). Growth inhibition of A-549/TE** cells at doses of 1 μM Gem was 15.5±1.6% greater than that of A-549 treated with 100 μM Gem. Similar findings were observed with Car, although the proliferation inhibition of A-549/TE* cells at doses of 50 μM Car was similar to the sum of effects of the separate drug and \(E\) gene treatments (Fig. 5). On the other hand, Pac induced a significant increase in proliferation...
inhibition when combined with the E gene (Fig. 5). The effect of the Pac 100 μM-E gene combination A-549/TE** (75.3±3.3) was greater than the sum of the separate effects of 100 μM Pac (44.7±2.4%) and continuous E gene transfection (19.7±2.0%) on A-549 cells. This enhancement of effects was also observed with this combined therapy at lower Pac concentrations. Thus, the lowest concentration of Pac 1 (μM) induced 46.6±2.5% and 58.8±3.1% proliferation inhibition in A-549/TE' and A-549/TE** cells, respectively, i.e., 1.9±0.4% and 14.2±1.4% greater proliferation inhibition than that obtained with 100 μM. Because the highest anti-proliferative effect was obtained with the combined E-gene-Pac therapy, the following experiments were conducted using this combination. To study the possible synergistic/additive effects of E gene and drug combination in colon cancer cells, T-84 cells were exposed to E gene transfection, to different concentrations of 5FU/FA/CPT-11 and 5FU/FA/Oxa alone, and in combination of both. The combined therapy of E gene and different concentrations of 5FU/FA/CPT-11 or 5FU/FA/Oxa were evaluated at different time-points. Results showed significant synergistic toxicity at 7 day, as compared to any agent alone (Figs. 6 and 7). Growth inhibition of T-84/TE** treated with 500:5:10 μM 5FU/FA/CPT-11 and 5:5:10 μM 5FU/FA/CPT-11 was 15±1.7% and 5.6±0.6%, respectively, greater than that of T-84 treated with 5000:5:200 μM 5FU/FA/CPT-11 (Fig. 6). The results revealed that the proliferation inhibition when using alone 5FU/FA/Oxa to the dose 5000:5:1000 μM (48.6±3.2%) was similar to the results
obtained to dose 500:5:0.5 μM (58.3±3.2%) and 5:5:10 μM (55.7±3.6%) in T-84/TE* (p<0.05) (Fig. 7). Because the highest anti-proliferative effect was obtained with the combined E gene-Pac (A-549) and E gene-5Fu/FA/Oxa (T-84) therapy, the following combined therapy experiments were conducted using this drug.

Apoptosis analysis. Significant differences in % apoptosis were found among control A-549 cells (0.7±0.1%), A-549/TE* cells (8.9±0.6%), cytotoxic drug-treated A-549 cells (100 μM) (73.4±4.2%) and cells receiving the E gene 1-μM Pac combined therapy (67±3.4%) (Fig. 8A). Similar results are shown in T-84 cells (0.1%), T-84/TE* cells (18±0.9%), cytotoxic drug-treated T-84 cells (5000:5:1000 μM) (38±2.7%) and cells receiving the E gene 500:5-0.5- and 5:5:10-μM 5Fu/FA/Oxa combined therapy (75.5±3.3 and 3±0.5%, respectively) (Fig. 8B). Treatment with empty vector had no significant apoptotic effect on these cells.

Discussion

Although in recent years there has been a progress in the development of new therapies and an improvement in the traditional treatments, the prognosis for patients with advanced colon or lung cancer remains poor. Unfortunately, the habitually late diagnosis of these tumours alongside the
ineffective and harmful effects of the conventional adjuvant treatments are responsible for the failure in the long-term survival increase after surgical resection. Thus, research related to the association of some anticancer strategies, including gene therapy and antitumour drugs, is necessary (22).

There is strong evidence that gene therapy can increase the efficiency of cytotoxic drugs in cancer treatment. In colon cancer, as shown recently by Yuan et al (23), the use of a combined therapy with mPNAS-4, a novel pro-apoptotic gene, associated to cisplatin led to an increase in tumour cell apoptosis in vitro and in vivo. The ability to target specific genes to cancer cells by promoters makes gene therapy a powerful tool to improve the response of the patient. The use of the urokinase plasminogen activator receptor (uPAR) promoter has been successfully used in colon cancer by the implication of the RAS signaling pathway in their carcinogenesis processes (24). Other authors (25) have successfully tested the carcinoembryonic antigen promoter (CEA)-cytosine deaminase gene expression for colon cancer treatment. Recently, He et al (26) demonstrated that the use of an AAV-mediated human interferon ß (IFN-ß) gene driven by hTERT promoter decreased colorectal and lung cancer cell viability, but not normal cells. In addition, this strategy induced tumor cell apoptosis mediated by the activation of caspase and by the release of cytochrome c in colorectal and lung cancer in mice resulting in tumor cells death in vivo. Research in lung cancer aims to improve the

Figure 7. Combined therapy (cytotoxic drugs and E gene transfection) in T-84 cells. Cytotoxic drugs 5FU:FA:Oxa were used at the indicated concentrations in T-84 parental cells, T-84/TE' and T-84/TE''. Values at 3, 5 and 7 days represent means ± SD of quadruplicate cultures.
outcome of the gene therapy strategies. Recent studies showed that NF-κB inducing kinase (NIK) is aberrantly expressed at the pre-translational level in NSCLC cell lines. Depletion of NIK by RNA interference remarkably diminished nuclear NF-κB DNA binding activity and reporter gene expression. These results indicate that NIK plays a key role in constitutive NF-κB activation in NSCLC cells and implicate NIK as a molecular target for lung cancer therapy (27). On the other hand, Sher et al. (28) have developed a specific promoter amplification system, called VISA (VP16-GAL4-WPRE integrated systemic amplifier) to enhance the surviving promoter activity (highly expressed in many

Figure 8. Apoptosis induced by combined therapy with E gene and cytotoxics in A-549 and T-84 cells. (A) Representative FACScan showing apoptosis in A-549 cells after combined therapy at 3 day. (a) Parental A-549 cells; (b) A-549/TE'; (c) A-549-Pac 100 μM; (d) A-549/TE'-Pac 1 μM. (B) Representative FACScan showing apoptosis in T-84 cells after combined therapy at 3 day. (a) Parental T-84 cells; (b) T-84/TE'; (c) T-84-5FU/FA/Oxa 5000:5:10 μM; (d) T-84/TE'-5FU/FA/Oxa 50:5:0.5 μM; (e) T-84/TE'-5FU/FA/Oxa 5:5:10 μM. These data are mean results of four separate experiments. Values represent means ± SD of quadruplicate cultures. Level of significance compared to control cells; *p<0.05.
cancers but not in normal adult tissues) in lung cell lines in vitro and in lung tumor tissue in vivo. Since survivin is a cancer specific promoter, the amplification from VISA made the activity level of survivin-VISA vector comparable to CMV promoter in lung cancer cells, but in normal cells, survivin-VISA was still much weaker than that of the CMV promoter. Combining the cancer-specific promoter with the VISA system might enable novel strategies to treat different cancers without toxicity to normal human tissues.

In this context, we have previously demonstrated that gef gene, a gene which does not need a prodrug, showed antitumour activity in breast cancer cells by inducing apoptosis (29), and that this gene is able to enhance the effect of drugs in lung cancer cells (11). Recently, we have demonstrated that a bacteriophage gene (E gene) has also antitumour activity (17). Interestingly, we have demonstrated this gene is able to reduce the chemotherapy dose classically used in the treatment of colon and lung cancer. Now, we are investigating the regulation of its expression through promoters in order to achieve a specific activity in different types of tumors. Using a eukaryotic expression vector (pcDNA3.1) (30,31), E gene was expressed in A-549 and T-84 cells which showed a clear decrease in cell growth rate. The largest reduction in proliferation was obtained with continual transfection of the E gene and after the longest period (7 days). The in vitro experiments designed to investigate whether E gene could induce apoptosis of A-549 and T-84 cells were positive. Because apoptosis may occur via either death-receptor-dependent (extrinsic) or mitochondrial (intrinsic) pathways we carried out experiments to determine the specific activate apoptosis pathway in colon and lung cancer cells. The apoptotic mitochondrial pathway is mediated by Bcl-2 family proteins which disrupt the mitochondria membrane potential and result in the release of apoptogenic factors (32). Transfection of A-549 and T-84 cells with E gene induced modulation of mitochondrial membrane potential, activation of caspasas-9 and -3 and cytochrome c release to cytoplasm suggesting that E gene induced mitochondria-mediated apoptosis. The experiments of Hengartner et al (33) confirm that the apoptosis by activation of mitochondrial pathway induces morphologic alterations. Our morphologic analysis in transfected cells supports the hypothesis of the mitochondrial gene induced mitochondria-mediated apoptosis. The gene could be fully active. To increase the anti-tumour effect of therapeutic drugs for the treatment of human lung and colon cancer. E gene was able to induce the apoptotic mitochondrial pathway in A-549 and T-84 cells. In addition, combined E gene/drug therapy enhanced cell growth inhibition of these cancer cells. Furthermore, the utility of this combined therapy suggests that E gene may be a new candidate for an effective strategy for lung and colon cancer treatment, specially for patients with a poor prognosis.

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References

GENE-CYTOTOXIC COMBINED THERAPY IN LUNG AND COLON CANCER


