Final Report

(6 months)

Title:

Effectiveness of anti-cancer extract against lung cancer

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Aim

A new extract was found to be effective against GI tract cancer cells and we wanted to check whether these extracts can be active against various cancer cells. Therefore, we tried to make small particles using either Jet-mill or Spray-drying technique and try to characterize these particles and then it will be used against various cancer cells.

Introduction

Cancer is a frightful disease and represents one of the biggest health-care issues for the human race and demands a proactive strategy for cure. Plants are reservoirs for novel chemical entities and provide a promising line for research on cancer. Hitherto, being effective, chemotherapy is accompanied by certain unbearable side effects. Nevertheless, plants and plant derived products are a revolutionizing field as these are simple, safer, eco-friendly, low-cost, fast, and less toxic as compared with conventional treatment methods.

Nearly 40% of lung cancers are adenocarcinoma, which usually originates in peripheral lung tissue. Although most cases of adenocarcinoma are associated with smoking, adenocarcinoma is also the most common form of lung cancer among people who have smoked fewer than 100 cigarettes in their lifetimes ("never-smokers") and ex-smokers with a modest smoking history. A subtype of adenocarcinoma, the bronchioloalveolar carcinoma, is more common in female never-smokers, and may have a better long-term survival.

Squamous-cell carcinoma accounts for about 30% of lung cancers. They typically occur close to large airways. A hollow cavity and associated cell death are commonly found at the center of the tumor. About 9% of lung cancers are large-cell carcinoma. These are so named because the cancer cells are large, with excess cytoplasm, large nuclei and conspicuous nucleoli.

This Respiratory symptoms of lung cancer are coughing, coughing up blood, wheezing, weight loss, weakness, fever, or clubbing of the fingernails and finally chest pain, bone pain, superior vena cava obstruction, or difficulty swallowing.

If the cancer grows in the airways, it may obstruct airflow, causing breathing difficulties. The obstruction can lead to accumulation of secretions behind the blockage, and predispose to pneumonia.

Many of the symptoms of lung cancer (poor appetite, weight loss, fever, fatigue) are not specific. In many people, the cancer has already spread beyond the original site by the time they have symptoms and seek medical attention. Symptoms that suggest the presence of metastatic disease include weight loss, bone pain and neurological symptoms (headaches, fainting, convulsions, or limb weakness). Common sites of spread include the brain, bone, adrenal glands, opposite lung, liver, pericardium, and kidneys. About 10% of people with lung cancer do not have symptoms at diagnosis; these cancers are incidentally found on routine chest radiography.

Similar to many other cancers, lung cancer is initiated by activation of oncogenes or inactivation of tumor suppressor genes. Carcinogens cause mutations in these genes which induce the development of cancer.

Mutations in the *K-ras* proto-oncogene are responsible for 10–30% of lung adenocarcinomas. About 4% of non-small-cell lung carcinomas involve an EML4-ALK tyrosine kinase fusion gene.

The epidermal growth factor receptor (EGFR) regulates cell proliferation, apoptosis, angiogenesis, and tumor invasion. Mutations and amplification of EGFR are common in non-small-cell lung carcinoma and provide the basis for treatment with EGFR-inhibitors. Her2/neu is affected less frequently. Other genes that are often mutated or amplified are *c-MET*, *NKX2-1*, *LKB1*, *PIK3CA*, and *BRAF*.

Metastasis of lung cancer requires transition from epithelial to mesenchymal cell type. This may occur through activation of signaling pathways such as Akt/GSK3Beta, MEK-ERK, Fas, and Par6.

Metastasis

Typical Napsin-A and TTF-1 immunostaining in primary lung carcinoma[1]

Histological type	Napsin-A	TTF-1
Squamous-cell carcinoma	Negative	Negative
Adenocarcinoma	Positive	Positive
Small-cell carcinoma	Negative	Positive

The lung is a common place for the spread of tumors from other parts of the body. Secondary cancers are classified by the site of origin; e.g., breast cancer that has spread to the lung is called metastatic breast cancer. Metastases often have a characteristic round appearance on chest radiograph.

Primary lung cancers themselves most commonly metastasize to the brain, bones, liver and adrenal glands. Immunostaining of a biopsy is often helpful to determine the original source. The presence of Napsin-A, TTF-1, CK7 and CK20 are helpful in confirming the subtype of lung carcinoma. SCLC derived from neuroendocrine cells may express CD56, neural cell adhesion molecule, synaptophysin or chromogranin.

Staging

Lung cancer staging is an assessment of the degree of spread of the cancer from its original source.[72] It is one of the factors affecting the prognosis and potential treatment of lung cancer.

The evaluation of non-small-cell lung carcinoma (NSCLC) staging uses the TNM classification. This is based on the size of the primary **t**umor, lymph **n**ode involvement, and distant **m**etastasis.

TNM classification in lung cancer[1][73]

T: Primary tumor			N: Lymph nodes		M: Metastasis	
TX	Any of:	Primary tumor cannot be assessed	NX	Regional lymph nodes cannot be assessed	MX	Distant metastasis cannot be assessed

		Tumor cells present in sputum or bronchial washing, but tumor not seen with imaging or bronchoscopy
T0	No evidence of primary tumor	
Tis	Carcinoma in situ	
T1	Tumor size less than or equal to 3 cm across, surrounded by lung or visceral pleura, without invasion proximal to the lobar bronchus	
T1a	Tumor size less than or equal to 2 cm across	
T1b	Tumor size more than 2 cm but less than or equal to 3 cm across	
T2	Any of:	Tumor size more than 3 cm but less than or equal to 7 cm across
		Involvement of the main bronchus at least 2 cm distal to the carina
		Invasion of visceral pleura

N0	No regional lymph node metastasis	
N1	Metastasis to ipsilateral peribronchial and/or hilar lymph nodes	
N2	Metastasis to ipsilateral mediastinal and/or subcarinal lymph nodes	
N3	Any of:	Metastasis to scalene or supraclavicular lymph nodes
		Metastasis to contralateral hilar or mediastinal lymph nodes

M0	No distant metastasis	
M1a	Any of:	Separate tumor nodule in the other lung
		Tumor with pleural nodules
		Malignant pleural or pericardial effusion
M1b	Distant metastasis	

		Atelectasis/obstructive pneumonitis extending to the hilum but not involving the whole lung		
T2a	Tumor size more than 3 cm but less than or equal to 5 cm across			
T2b	Tumor size more than 5 cm but less than or equal to 7 cm across			
T3	Any of:	Tumor size more than 7 cm across		
		Invasion into the chest wall, diaphragm, phrenic nerve, mediastinal pleura or parietal pericardium		
		Tumor less than 2 cm distal to the carina, but not involving the carina		
		Atelectasis/obstructive pneumonitis of the whole lung		
		Separate tumor nodule in the same lobe		
T4	Any	Invasion of the mediastinum, heart, great vessels, trachea,		

	of:	carina, recurrent laryngeal nerve, esophagus, or vertebra		
		Separate tumor nodule in a different lobe of the same lung		

Using the TNM descriptors, a group is assigned, ranging from occult cancer, through stages 0, IA (one-A), IB, IIA, IIB, IIIA, IIIB and IV (four). This stage group assists with the choice of treatment and estimation of prognosis.

Stage group according to TNM classification in lung cancer

TNM	Stage group
T1a–T1b N0 M0	IA
T2a N0 M0	IB
T1a–T2a N1 M0	IIA
T2b N0 M0	
T2b N1 M0	IIB
T3 N0 M0	
T1a–T3 N2 M0	IIIA
T3 N1 M0	
T4 N0–N1 M0	
N3 M0	IIIB
T4 N2 M0	
M1	IV

Small-cell lung carcinoma (SCLC) has traditionally been classified as "limited stage" (confined to one-half of the chest and within the scope of a single tolerable radiotherapy field) or

"extensive stage" (more widespread disease). However, the TNM classification and grouping are useful in estimating prognosis.

For both NSCLC and SCLC, the two general types of staging evaluations are clinical staging and surgical staging. Clinical staging is performed prior to definitive surgery. It is based on the results of imaging studies (such as CT scans and PET scans) and biopsy results. Surgical staging is evaluated either during or after the operation and is based on the combined results of surgical and clinical findings, including surgical sampling of thoracic lymph nodes.

This current plant extract which acts selective in their functions and functions specifically on tumor cells without affecting normal cells. This extract is considered suitable candidates for anticancer drug development due to their pleiotropic actions on target events with multiple manners. The primary research showed the current extract can block or slow down the growth of cancer cells without any side effects.

The practical experiments

The FDA drug development process is listed below. This plant extract has passed step 1 and step 2 and the results are available. However, for further studies on Lung cancer it needs to classify the database specifically on Step 2. For drug approval we need to pass step 3 and classify the data either.

Step 1: Discovery and Development

Discovery

Typically, researchers discover new drugs through:

- New insights into a disease process that allow researchers to design a product to stop or reverse the effects of the disease.
- Many tests of molecular compounds to find possible beneficial effects against any of a large number of diseases.

- Existing treatments that have unanticipated effects.
- New technologies, such as those that provide new ways to target medical products to specific sites within the body or to manipulate genetic material.

Development

Once researchers identify a promising compound for development, they conduct experiments to gather information on:

- How it is absorbed, distributed, metabolized, and excreted.
- Its potential benefits and mechanisms of action.
- The best dosage.
- The best way to give the drug (such as by mouth or injection).
- Side effects or adverse events that can often be referred to as toxicity.
- How it affects different groups of people (such as by gender, race, or ethnicity) differently.
- How it interacts with other drugs and treatments.
- Its effectiveness as compared with similar drugs.

Step 2: Preclinical Research

- The below in vitro studies will be considered for our practical 6 months period:

    - ✓ **Making Mirco(nano)particles**
    - ✓ **Particle Size Evaluations and Measurements**
    - ✓ **Performing Cell studies**

Making Micro(nano)particles

We used two techniques to compare the particles shape and size, one spray-drying and the other one jet-milling.

Jet-Milling

Compressed gas was forced into the mill through nozzles tangent to the cylinder wall, creating a vortex while 50 mg of plant extract was placed in the mill. The gas leaves the mill through a tube along the axis of the cylinder. Solid particles in the mill were subject to two competing forces:

1. Centrifugal force created by the particles traveling in circles
2. Centripetal force created by the drag from the gas as it flows from the nozzles along the wall to the outlet in the center of the mill

The particles will be pulled toward the wall of the mill according to the square of their radius or diameter. Large particles will continue the comminution process, until they are small enough to stay in the center of the mill where the discharge port is located.

Spray-drying

The plant extract was dissolved in 500 ml of ethanol and DMSO. The solution was spray-dried using a Mini Spray-Dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland). The spray drying was done in a tall type spray drier under 2 different temperatures 150 °C and 170 °C. The spray drying condition for was set as inlet air temperature of 150 °C and 170 °C, outlet air temperature of 80 ± 10 °C and 105 ± 10 °C, respectively and a flow rate of 60%.

Particle Size Evaluations and Measurements

The obtained particles using both Jet-mill and Spray-drying were evaluated based on the following methods:

- Dynamic laser spectroscopy

Photon correlation spectroscopy and laser doppler anemometry were applied to measure particle size and zeta potential, respectively. Utilizing a zetasizer 3000hs (Malvern Instruments, Malvern, UK) at 25 °C, the particle size distribution was reported as PdI. All the tests were performed in triplicate, and the responses were reported as mean ± SD.

- Scanning Electron Microscopy

The nanoparticles morphology was studied by scanning electron microscopy (SEM) using SEM Hitachi, S4160 (Tokyo, Japan). Briefly, the nanoparticles were diluted in water and sprayed on an aluminum disc. After drying at room temperature, the particles were coated with gold using sputter-covered for 10 min at 6 mA and 15 KV(DC).

Cell culture study

Briefly, cells were seeded at 1×10^5 cells/mL in 96 well microtiter plates in Minimum Essential Medium with fetal bovine serum. The cells were incubated overnight for attachment. Drug concentrations in serial four-fold dilutions (0mg/ml, 1mg/ml, 2mg/ml and 4mg/ml) were added and incubated for 48h at 5% CO_2 at 37°C. Thereafter, the cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT). Four hours later, all of the medium including MTT solution (5 mg/mL) was aspirated from the wells. The cell counts and cell viability were recorded for listed cell lines as below:

MCF-7: Human breast adenocarcinoma cell line.

Hep-2: Human cervical cancer cell line.

Hep-G2: Human liver cancer cell line.

Saos-2: Human osteosarcoma cell line.

MRC-5: Cell line human (control).

Stem cell (control).

Results and Discussion

The obtained results showed the particles which were prepared using Jet-mill at room temperature resulted in a size of about 5 micron whereas the particles which were jet-milled by first placed in liquid nitrogen were about 3 micron; and those which were prepared by spray drying were about 300 nm.

This is verified by SEM where in Figure 1, we can see the particles of jet-milled and in Figure 2 we can see the particles obtained from spray drying.

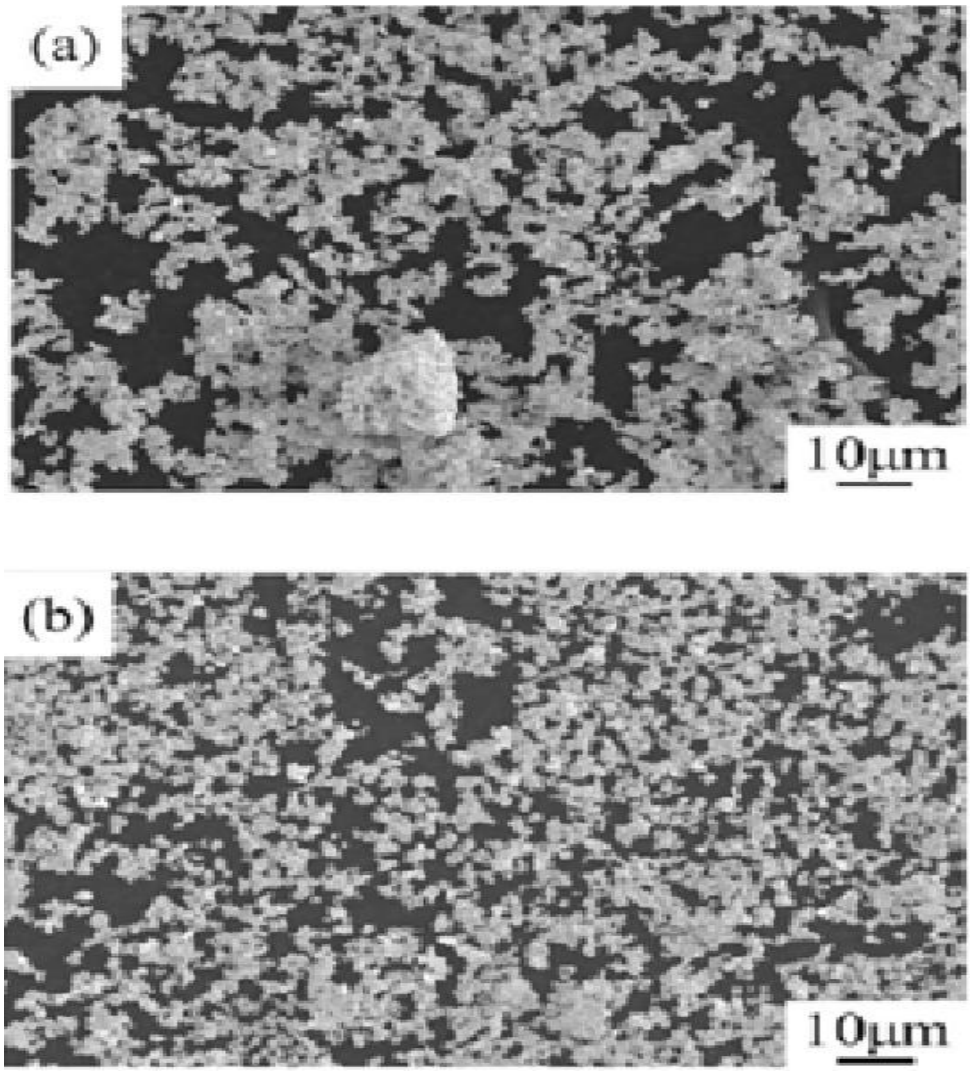

Figure 1- SEM of Jet-milled powders: a) extract powder at room temperature; b) soaked extract in liquid nitrogen and then jet-milled

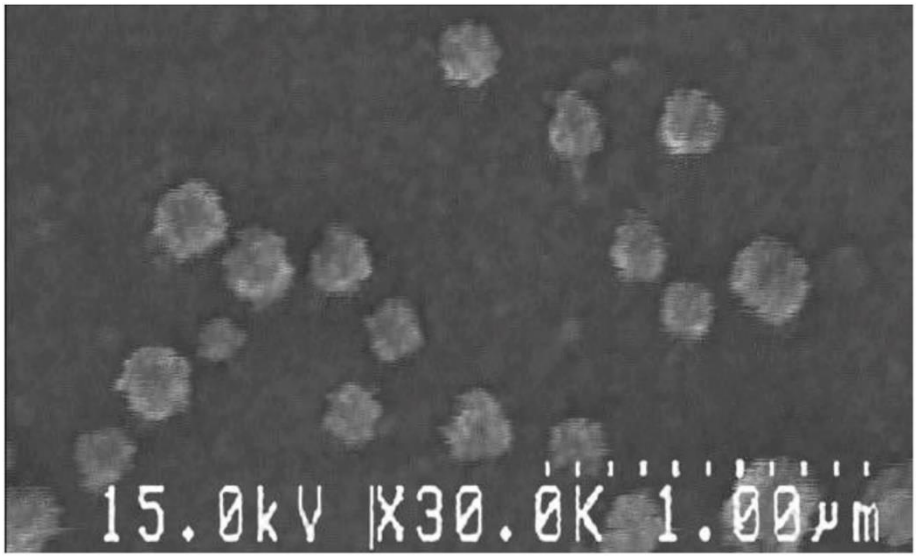

Figure 2- SEM of spray-dried products

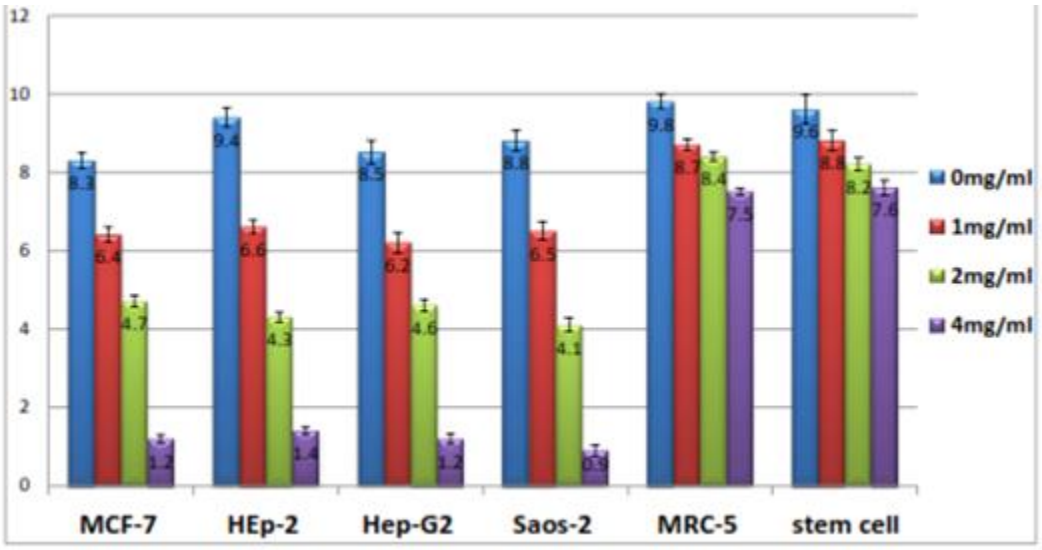

Figure 3- Effect of extract on cell counts. The Saos-2, MCF-7 and HEp-2 in comparison to control cell lines (Stem cell and MRC-5). All the results were observed in 48 h.

As it is observed in Figure 3, with the change of concentration from 0 to 4 mg/ml, the effectivity of the extract for cell death will be also higher.

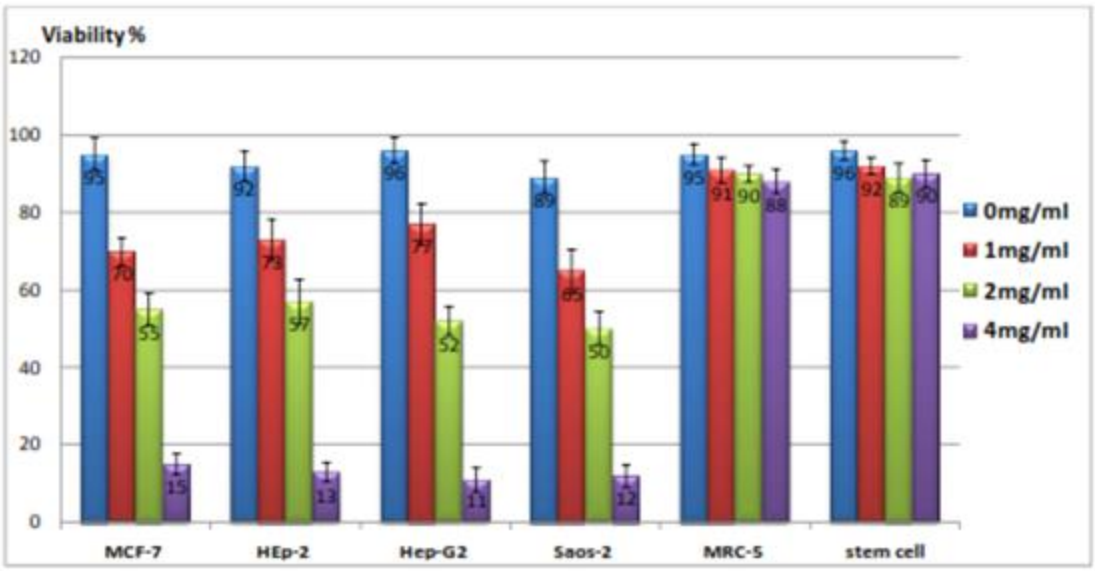

Figure 4- Effect of extract on cell viability of Saos-2, MCF-7 and HEp-2 in comparison to control cell lines (Stem cell and MRC-5). All the results were observed in 48 h.

As it is observed in Figure 4, with the change of concentration from 0 to 4 mg/ml, the cell viability will be less which shows a linear effectivity-concentration correlation.

The cytotoxicity index was determined using the untreated cells as negative control. The percentage of cytotoxicity was calculated using the background-corrected absorbance follows:

Figure 5- Cytotoxicity effect after 48 hours.

% cytotoxicity=(1−absorbance of experimental well)/(absorbance of negative control well)

The extract cytotoxic results has been investigated on the LN-Cap(human prostate cancer cell line) in comparison with C2Cl2(normal mouse myoblast cell line) as control. The data in Figure 5 shows the higher cytotoxicity in LN-Cap compared to C2C12.

Conclusion

The current study shows that our new extract is quite effective in various cell lines and we can expect to be considered as a promising medicine for treatment of cancer cells.

Other activities

- Establishing new possible collaboration with other staff here at King's College
- Taking part in various scientific sessions

Attending various lectures given by many invited lecturers or professors from King's college or other universities.