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Study on cancer stem cell labeling and inhibition efficiency of LV3 nanocomplex *in vitro*

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Abstract:

Cancer stem cells (CSCs) are the cancer cells that have abilities to self-renew, differentiate into defined progenies, and to initiate and maintain tumour growth. They also contribute to cancer metastasis and therapeutic resistance, both of which are the principal causes of cancer mortality. Therefore, finding efficient fluorescent materials for CSC labelling and basic research is an urgent need. Thus, this research is focused on using a rare-earth element, which is the fluorescent Tb^{3+} nano-ion, and the CD133 monoclonal antibody to create a CSC-targeting nanocomplex (LV3). Tb^{3+} nanorods were silica-surface treated and $-NH_2$ activated before being conjugated with the monoclonal antibody (mAb) against CD133, a typical CSC surface marker. The use of robust fluorescent Tb^{3+} nanorods was to decrease the toxicity of a high-dose prober while the CD133 mAb would increase the CSC's specific binding capacity of the LV3. The fluorescent properties of the coupled LV3 complex were measured and CSC-targeting label activities on the pluripotent human embryonal carcinoma cell line (NTERA-2) were observed. The obtained results presented fluorescent images of LV3 exposed to NTERA-2 cells under microscopy. LV3 also demonstrated that it effectively labelled up to 99.68% of the tested NTERA-2 cells. By contrast, LV3 only labelled 1.44% of the CCD-18Co human healthy cells. On the other hand, LV3 exhibited anti-CSC activity, which inhibited 11.14% *in vitro* and 30.5% tumourspheroid growth of NTERA-2 cells. In conclusion, LV3 showed its efficiency in specific CSC target labelling and inhibition, which could be further applied to fundamental and preclinical research.

Keywords: **cancer stem cells, CCD-18Co, CD133 monoclonal antibody, ion Tb^{3+}, LV3, NTERA-2.**

Classification number: **3.2**

Introduction

Despite abundant ongoing research efforts, cancer remains one of the most challenging diseases to treat globally. Due to the heterogeneous nature of cancer, one of the major clinical challenges is the ability of cancer to develop resistance in therapeutic development. It has been hypothesized that cancer stem cells (CSCs) are the cause of this resistance and targeting their treatment will lead to tumour regression [1]. CSCs accounts for a small percentage of tumours and can regenerate into various tumorous cell types causing the growth and expansion of malignancy. CSCs present drug-resistant abilities and overcome radiotherapy. Then, the survival of cancer stem cells after treatment allows the tumour to recur and spread throughout the body. Therefore, CSCs are considered a promising target for research and discovery of more effective anticancer drugs or therapies. CSCs are characterized by several specific surface markers. A pentaspan transmembrane glycoprotein, CD133, has been suggested to mark cancer stem cells in various tumour types. However, the accuracy of CD133 as a cancer stem cell biomarker has been highly controversial [1]. CD133 is known as prominin-1, a transmembrane glycoprotein, and is a common surface marker for CSCs, which are inside of various cancer tumours. This transmembrane CD133 glycoprotein includes an extracellular N-terminus and an intracellular C-terminus, which have been used as an efficacious typical surface antigen to detect and to isolate CSCs [2]. As recognized, traditionally nanotechnological biomedicine heighten pharmaceutical properties and reduce the systemic toxicity of chemotherapy through selectively targeting and effectively transferring anticancer drugs to tumours. Nanoparticles usually improve the therapeutic index of the chemotherapeutic drugs that are enveloped inside or combined with the nanoparticle surfaces. For

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example, rare-earth elements such as Tb^{3+} nano-materials or cation terbium (III) emitting green fluorescence would effectively assist in detection and treatment [3]. Thus, experimental and clinical applications of CSC labelling and tracking are interesting to evaluate cell location. Rare-earth-based nanotechnology would be very helpful [4].

Also, several studies on nanomaterial labelling effects targeting cancer cells have been reported elsewhere [5, 6]. According to the previous report from the authors in this study [7], Tb^{3+} a rare-earth ion could constitute a fluorescent nanomaterial as terbium orthophosphate monohydrate ($TbPO_4.H_2O$) in the form of hexagonal crystal structure. Fluorescent spectra of $TbPO_4.H_2O$ nanomaterials at pH=2, incubated at 200°C for 24 h, coated by silica, and functionalized with $-NH_2$ measured at 355 nm was determined by iHR55 system (Jobin-Yvon). The fluorescent images of human colorectal adenocarcinoma cells (HT-29) were observed by the effects of the complex of the surface-functionalized $TbPO_4.H_2O$-NH_2 and the anti-CD133 mAb [7]. Our previous study also reported that the fluorescent Tb^{3+} nano-ion and CD133 mAb conjugation (ET complex) gave marks on 26.89% (of colorectal cancer cells) [8] and 97.74% (of NTERA-2 cells) compared to the control, respectively [9]. To continue this study, we modify the content of one component of the RT2 [9] complex to make LV3 and attempted to evaluate the CSC growth inhibition activities of the LV3 complex.

Material and method

Materials

LV3 is made from nano Tb^{3+}-monoclonal antibody (RT) transport system [9].

The pluripotent human embryonal carcinoma cell line (NTERA-2) and the human healthy cell line (CCD-18Co) were kindly provided by Dr. P. Wongtrakoongate, Mahidol University, Thailand, and Prof. Chi-Ying Huang, National Yang-Ming University, Taiwan. Cells were maintained in DMEM medium supplement with 10% foetal bovine serum and 1% antibiotics (antibiotics-antimycotics solution, Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO_2 at 37°C.

Cultured medium so-called Dulbecco's Modified Eagle Medium (DMEM), Foetal bovine serum (FBS), Trypsin-EDTA, antibiotics (antibiotics-antimycotics) were purchased from Invitrogen (Carlsbad, CA, USA). Human CD133 monoclonal antibody and human CD133 antibody conjugated with FITC (FITC-CD133) were from Miltenyi Biotec (Bergisch Gladbach, Germany). Other chemicals were provided by Sigma Aldrich (St. Louis, MO, USA).

In vitro cell culture

The *in vitro* cell culture was carried out by following the protocols from ATCC Cell Bank (American Type Culture Collection, USA). Accordingly, NTERA-2 and CCD-18Co cells were cultured in T75 flask with DMEM supplemented by 2 mM L-glutamine, 10% foetal bovine serum (FBS), and 1% antibiotic (Anti-Anti solution). The cells were subcultured every 3-5 d with a ratio of 1:3 and incubated in humid conditions of 37°C and 5% CO_2.

Labelling cells with LV3

- Cancer stem cells and healthy cells imaging: using LV3: NTERA-2 and CCD-18Co, cells were pre-seeded into a 96-well plate at 10,000 cells/well and incubated at 37°C, 5% CO_2 for 24 h. Then, the culture medium was replaced with 10% formaldehyde to fix the cells for 10 min at room temperature. The cells were triple rinsed with phosphate buffered saline (PBS) to thoroughly remove formaldehyde. Then, 10 µl of LV3 in 190 µl of PBS were placed into each well and incubated at 4°C for 1 h. The unbound sample was removed and triple rinsed with PBS. Finally, 100 µl PBS was added to the wells before observation using an Olympus Scan^R fluorescence microscope (Olympus Europa SE & Co.KG, Hamburg, DE).

- Determining the number of marked cells (through CD133 surface marker) by flow-cytometry: cancer stem cells (NTERA-2) and healthy cells (CCD-18Co) were seeded into a 6-well plate and incubated at 37°C, 5% CO_2 overnight. After 24 h of incubation, the cells were harvested with trypsin-EDTA and collected into a falcon tube. Cells were re-suspended with DMEM medium containing 2% FBS, LV3, or anti-CD133-FITC mAb and then incubated at 4°C for 10-15 min while protected from light. The number of labelled cells (out of 10000-12000 counting cells) were measured and analysed by Novocyte flow cytometry system (ACEA Bioscience Inc.) and NovoExpress software.

LV3 cytotoxic determination

The MTT assay was employed according to Mosmann (1983) [10] to measure the cytotoxic activity of the LV3 nanocomplex. In short, cells were seeded in 96-well plates and triplicated, then treated with LV3 at various concentrations for 72 h at 37°C, 5% CO_2. Then, 10 µl MTT (5 mg/ml) was added to each well and incubated at

37°C for 4 h. The medium was discarded and the formazan crystal was dissolved by using 50 µl/well dimethlysulfoxide (DMSO). The OD values were measured at 540 nm by a spectrophotometer (BioTek, ELx800). The number of survived cells was calculated by the formula:

$$\% \text{ survived} = \frac{OD(reagent) - OD(blank)}{OD(control) - OD\ (blank)} x100$$

Measurement of 3D tumoursphere growth inhibitive activities

BALB/c mice macrophages were isolated using a Macrophage Mouse Isolation Kit (Peritoneum) (Miltenyi Biotech., Bergisch Gladbach, Germany). The isolated cells were cultured in DMEM medium containing 10% FBS and 1% antibiotics at 37°C and 5% CO_2.

In order to form tumour spheroids, the hanging drop method was performed. The 1500 NTERA-2 cells in 20 µl of medium were dropped onto the bottom of the 60 mm tissue culture dish lid before inverting that lid onto the 5-ml medium filled bottom dish. The dish was then incubated at 37°C, 5% CO_2, and 95% humidity. After 3-d incubation, cell aggregates formed. These 3D tumourspheres were further co-cultured with macrophages in a 96-well plate. Wells were covered with 1% agarose before the spheroids were transferred to the wells. The macrophage cells were then co-cultured with the spheroids in the wells. The LV3 treatment was performed by directly adding LV3 into the co-culture wells and further incubated for 3 d. The growth of the spheroids was observed under microscopy. The images were analysed using ImageJ software.

Statistical analysis

The data was reported as mean±standard deviation (SD) and analysed by the GraphPad Prism 7 software using an unpaired *t*-test. A $p<0.05$ was considered statistically significant.

Results and discussion

Probing NTERA-2 and CCD-18Co cells and with LV3 fluorescent nanocomplex

The results exhibited that the NTERA-2 cells were labelled by LV3 and displayed strong fluorescence under fluorescence microscopy (Fig. 1, Table 1). The healthy cells known as CCD-18Co did not emit any corresponding signal under the same condition (Fig. 2). Therefore, LV3 could be specifically targeting cancer stem cells. The obtained results were consistent with the research of Le Nhat Minh, et al. (2019) [9].

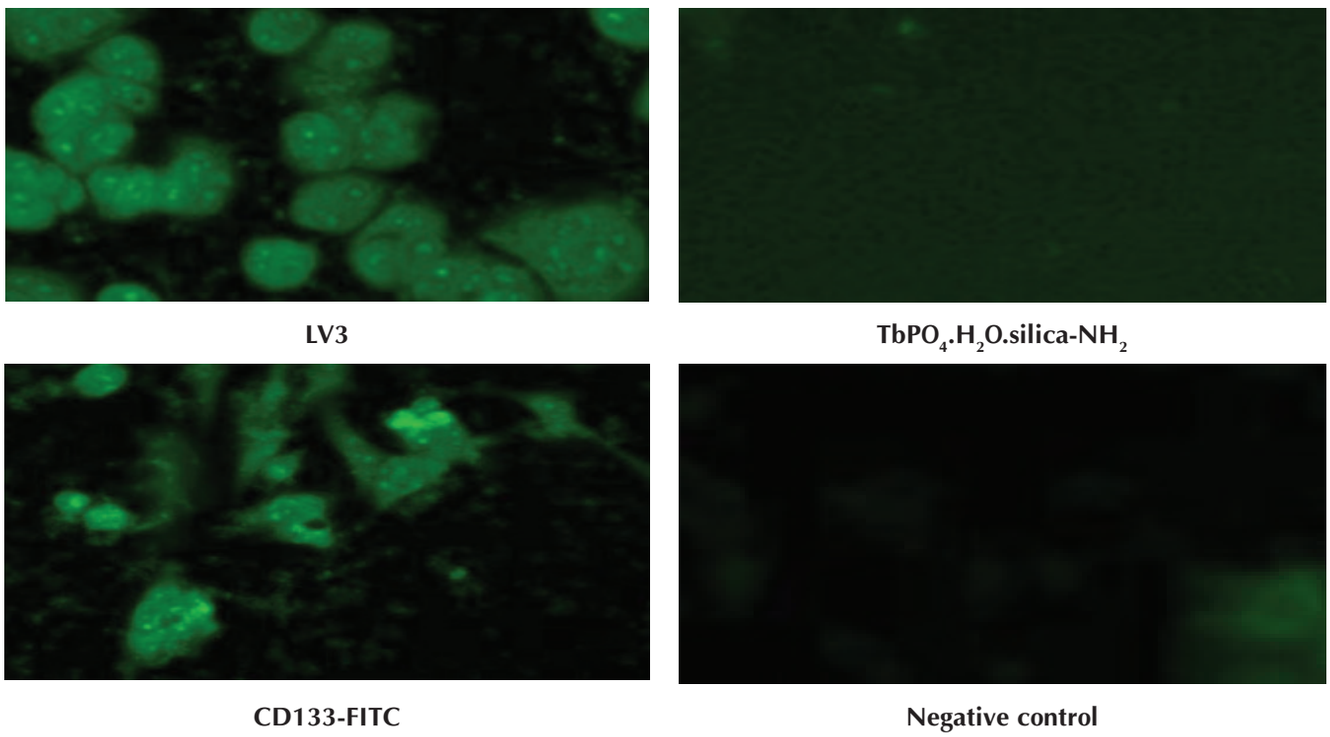


Fig. 1. NTERA-2 cells was probed after 1 h of incubation either with LV3, $TbPO_4.H_2O$.silica-NH_2, CD133-FITC, or without LV3 (negative control) by fluorescence microscopy on an Olympus Scan^R.

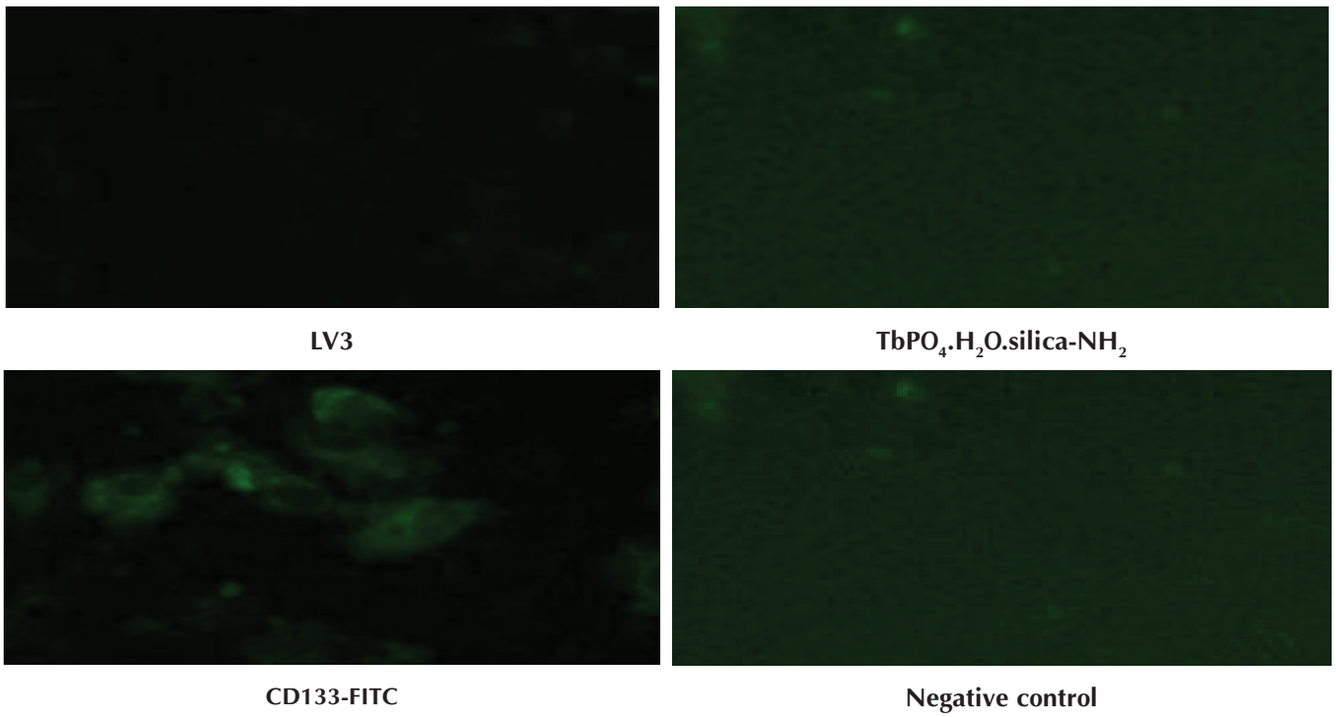


Fig. 2. CCD-18Co cells was probed after 1 h of incubation either with LV3, $TbPO_4.H_2O$.silica-NH_2, CD133-FITC, or without LV3 (negative control) by fluorescence microscopy on an Olympus Scan^R.

Table 1. Labelling performance of cancer stem cells and healthy cells.

Samples	The number of fluorescent detected cells (%)	
	Cancer stem cells (NTERA-2)	*Healthy cells (CCD-18Co)*
LV3	99.68±3.85	1.44±0.11
$TbPO_4.H_2O$.silica-NH_2	0.57±0.07	0.31±0.02
CD133-FITC	95.83±7.31	1.17±0.06
Negative control	0.10±0.02	0.20±0.04

Table 2. Total intensity of samples in examined cell lines.

Samples	Total intensity	
	NTERA-2	*CCD-18Co*
LV3	6011±62.62**	0
CD133-FITC	5497±42.87	0
Negative control	19.00±3.07	0

Negative control sample; LV3 - Experimental sample. Data is expressed as mean ± SE (n=3) combined from three repeated experiments. *Significant differences (t-test, p≤0.05) and **(p≤0.01).

The fluorescent intensity was analysed in the LV3-treated NTERA-2 cell line by fluorescence microscope as shown in Table 2. By fluorescence spectroscopy, NTERA-2 emits fluorescence intensity at 6011±62.62 FU, which is statistically significant compared to the negative control. Meanwhile, fluorescence intensity in healthy cells were not measurable. This result is consistent with the study of Le Nhat Minh, et al. (2019) [9] and is demonstrated in Fig. 1.

LV3 fluorescent labelling performance by flowcytometry evaluation

We used flow-cytometry to evaluate the labelling specificity of LV3. Detailed results are shown in Table 1 and Fig. 3.

The results showed that the LV3 probed 99.68% of NTERA-2 cells, which was higher than the respective number (0.10%) of the negative control. As a result, it is seen that LV3 labelled NTERA-2 cells more efficiently than the RT labelling of the colorectal cancer cell [9]. Further research is required to elucidate how LV3 could label a cancer stem cell better than in other cancer cell lines.

Besides, LV3 could not label and distinguish CCD-18Co. Thus, LV3 is an effective material for labelling CSCs. These preliminary results demand more studies *in vivo* and clinical testing.

Recent clinical studies have shown that high expression of CD133 in tumours plays an important role as a prognostic marker of disease progression. As such, a spectrum of immunotherapeutic strategies has been developed to target these CD133 positive cells with the goal of translation into the clinic. In one report, the researcher Mi Y used salinomycin-loaded poly (lactic-co-glycolic acid) - poly (ethylene glycol) nanoparticles conjugated with CD133 antibodies (CD133-SAL-NP) to eliminate $CD133^+$ ovarian

CSCs. The CD133-SAL-NPs efficiently bound to the $CD133^+$ ovarian cancer cells resulting in an increased cytotoxic effect toward $CD133^+$ ovarian cancer cells when compared with the untargeted SAL-NPs and salinomycin. The CD133-SAL-NPs reduced the percentage of $CD133^+$ ovarian CSCs in ovarian cells more effectively than treatment with salinomycin or SAL-NPs, suggesting that CD133-SAL-NP targeted $CD133^+$ ovarian CSCs [11].

Herein, rare-earth nano-ion Tb^{3+} conjugated with anti-CD133 mAb to formulate LV3 presents promising CSC labelling and specific targeting capacities. This might also be the first developing stage of rare-earth-based nanomaterials for valuable applications in cancer diagnostics and treatment.

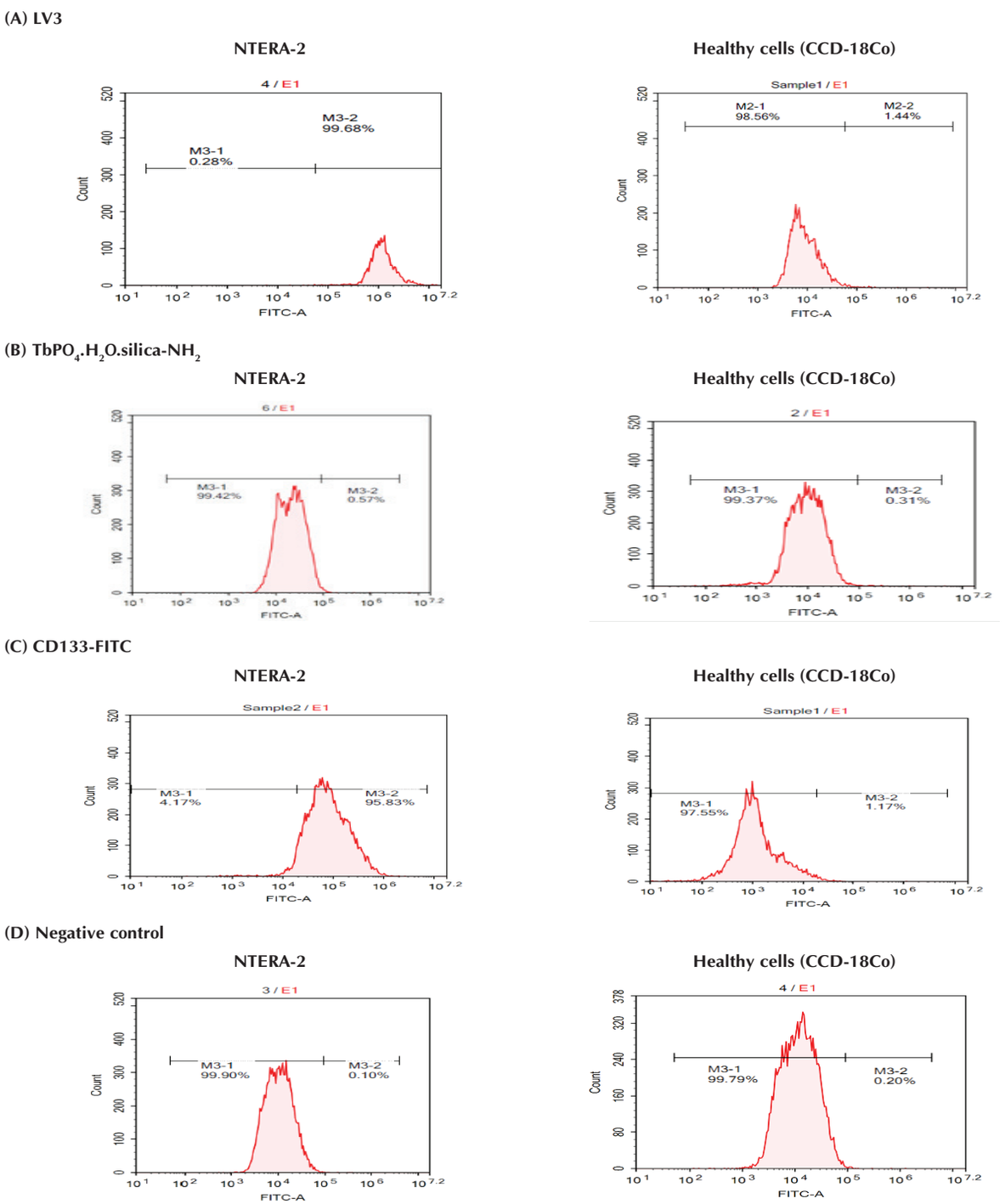


Fig. 3. Flow-cytometry analysis to determine the number of fluorescent labelled cells with various materials: (A) LV3, (B) $TbPO_4.H_2O$.silica-NH_2, (C) CD133-FITC, and (D) negative control.

Effect of LV3 on the proliferation of NTERA-2

The proliferation of LV3-treated NTERA-2 cell was assessed by using MTT assays. LV3 showed the ability to inhibit the growth of NTERA-2 cells up to 11.14% at a concentration of 10 µg/ml (Table 3). The anti-proliferation of LV3 on NTERA-2 cells was slightly higher than that on CCD-18Co cells.

Table 3. The proliferation of complex on NTERA-2 cells and CCD-18Co cells.

Samples	% proliferation	
	NTERA-2	*CCD-18Co*
LV3	88.86±2.13	95.05±0.68
Negative control	100	100

Effect of LV3 on NTERA-2 spheroids co-culture with macrophages

Although several CSCs markers have been reported, one of the most promising and possibly least ubiquitous is CD133, a frequently expressed surface marker on CSCs. Some evidence has indicated that directly targeting CD133 with biological drugs might eliminate CSCs effectively [12].

Table 4. 3D tumour spheroids inhibited under the treatment of LV3.

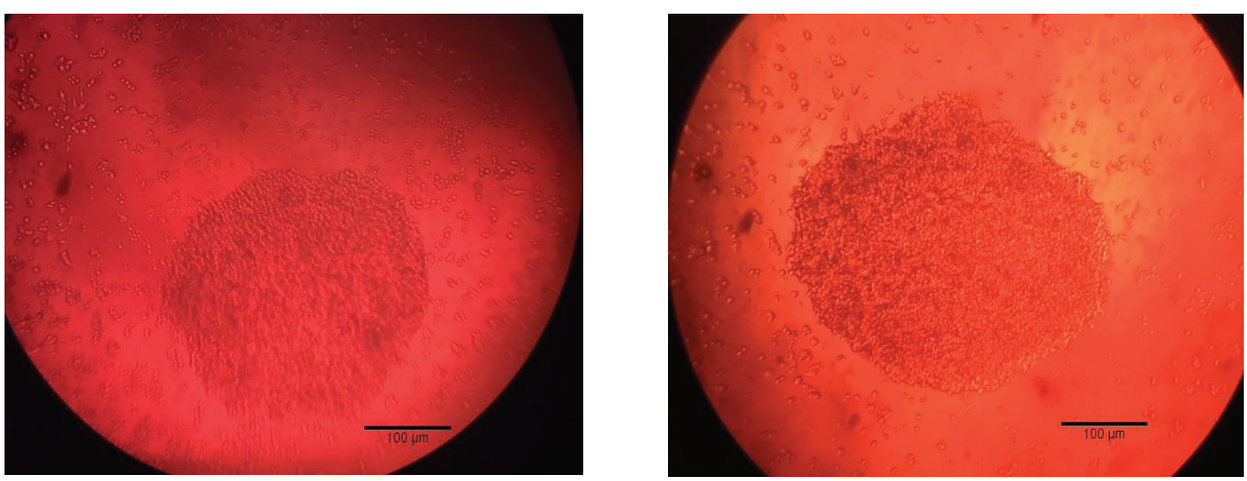
Samples	3D tumour spheroids (%)	Standard deviation
LV3	69.50*	0.81
Negative control	100.00	0.09

Negative control sample; LV3 - Experimental sample. Data is expressed as mean ± SE (n=3) combined from three repeated experiments. * Significant differences (t test, p≤0.05).

In this study, the activity of LV3 inhibited 3D-tumor growth *in vitro* formed by co-culturing 3D NTERA-2 spheroids with macrophages (Table 4; Fig. 4). As a result, the diameters of 3D spheroids dropped to 69.50% in comparison with that of the untreated negative control after a 3-d treatment. Herein, although LV3 slightly inhibited the growth of NTERA-2 cells (11.14%) *in vitro*, this nanocomplex strongly inhibited the growth of these 3D NTERA-2 spheroids (30.5%) when co-cultured with macrophages.

Among the reported markers of the cancer stem cells, CD133 is the most well-known marker for isolating and studying CSCs in different types of cancer. The CD133 high population of cancer cells are not only capable of self-renewal and proliferation but are also highly metastatic and resistant to therapy. Despite limited information on the physiological functions of CD133, many ongoing studies aim to reveal the mechanisms that CD133 utilizes to modulate cancer dissemination and drug resistance (Liou, 2019) [13]. Thus, the role of anti-CD133 antibodies in the LV3 may reduce the function of CD133 and result in the inhibition of CSCs.

According to another report that investigates the cytotoxic, radiation dose-enhancing, and radio-sensitizing ability of five rare-earth oxide nanoparticles on the two immortalized mammalian cell lines U-87 MG and Mo59K, a significant cytotoxicity of Nd_2O_3 and La_2O_3 was observed in U-87 MG cells. As aforementioned, the component of LV3 is terbium (Tb^{3+}), which is a typical lanthanide with green fluorescence that has the potential for biomedical labelling and imaging. Seemingly, terbium in LV3 is likely to have an inhibitory effect on CSCs. In this study, LV3 was



3D tumour treated with LV3 — 3D tumour – negative control

Fig 4. The 3D tumourspheres at the day 3 under the treatment of LV3 and negative control under the fluorescence microscope system Olympus Scan^R 100X.

demonstrated to be a promising target for drug delivery to CSCs and may be useful as an agent to inhibit the growth of cancer by targeting CSCs. LV3 may, therefore, represent a promising approach for the treatment of cancer.

Conclusions

LV3, which was a combination between the rare-earth-based Tb^{3+} nanorod and CD133 monoclonal antibody, was assessed for its fluorescent properties and tumoursphere inhibition using cancer stem cells (NTERA-2) and healthy human colon cells (CCD-18Co). The LV3-probed NTERA-2 cells exhibited strong emission under fluorescent microscopic observation. The NTERA-2 labelling efficiency of the LV3 was 99.68% from flow cytometric analysis whereas healthy cells (CCD-18Co) were weakly probed (1.44%). Also, LV3 was shown to be a promising anti-CSC factor in which 11.14% survived inhibition *in vitro* and 30.50% tumourspheroid inhibition of NTERA-2 cells. In conclusion, LV3 has presented as highly effective in targeting cancer stem cells *in vitro*.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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