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DIFFERENTIAL UP-TAKE AND FLUORESCENCE OF CURCUMIN, A YELLOW PIGMENT FROM TURMERIC, IN NORMAL VS TUMOR CELLS

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Abstract

Using absorption and fluorescence spectroscopic methods, quantitative cellular uptake of curcumin, was calculated in two types of normal cells: spleen lymphocytes and NIH3T3 and two tumor cell lines: EL4 & MCF7. Both the uptake and fluorescence intensity of curcumin were significantly higher in tumor cells as compared to the normal cells. Using laser confocal microscopy, intracellular localization of curcumin was monitored and the results indicated that curcumin is located both in the cell membrane and in the nucleus.

Introduction

Curcumin is a polyphenolic yellow pigment isolated from the rhizomes of *Curcuma longa* (turmeric), a medicinal plant widely used in traditional Indian and Chinese medicine [1]. In spite of great advancement on the therapeutic research of curcumin, there are still very few studies reporting the methodologies to quantitatively estimate intracellular curcumin uptake and localization. In the present study, cellular uptake measurements of curcumin in four different cell types, two normal cells, *viz.*, mouse spleen lymphocytes, NIH3T3 (mouse fibroblast cells), and two tumor cell lines, *viz.*, EL4 (T cell lymphoma of murine origin) and MCF7 (breast cancer cells of human origin). Following absorption spectra of the cell lysate and the fluorescence spectra of cellular curcumin, quantitative uptake was calculated. Further, following curcumin fluorescence by confocal microscopy, its intracellular localization was monitored.

Materials and Method

Chemicals and equipment

Curcumin, 4 '6-diamidino-2-phenylindol (DAPI), cell culture medium (RPMI 1640 & DMEM) were procured from the local agents**.** Mouse spleen lymphocytes were freshly isolated as given in reference [2] and EL4, MCF7 and NIH3T3 cell lines were obtained from National Centre for Cell Science (NCCS), India. JASCO V-530 spectrophotometer and Hitachi F-4010 fluorimeter were used, to record absorption and fluorescence spectra respectively. Solutions were prepared in nanopure water from a Millipore Milli-Q system.

Cell culture and uptake studies

Spleen, NIH3T3 & EL4 cell lines were cultured in suspension with RPMI medium and MCF7 cell line in DMEM medium. The treatment of cells with curcumin and then quantitative estimation of cellular curcumin for uptake studies was done as described in earlier reference [2].

Confocal microscopy and image analysis

Slides for confocal microscopy were prepared by fixing curcumin-loaded cells and then sealed with cover slip using mounting medium. Fluorescence imaging of cells was performed with an Olympus Fluoview 500 confocal laser-scanning microscope (Olympus, Tokyo, Japan) equipped with a multi-Argon laser for excitation at 458, 488 and 515 nm [3]. The images were acquired either with 20X objective or a 60X water immersion objective using the fluoview software (Olympus, Tokyo, Japan). The curcumin emission was collected using the barrier filter BA505. Fluorescence images of DAPI stained cells were excited using Coherent Mira 900F Titanium: Sapphire infrared laser (Coherent Verdi-V5, Santa Clara, CA, USA) tuned to 720 nm and the fluorescence in the range 400-470 was collected. At least five monochrome images were accumulated from five different microscopic fields of the same slide. The desired region on each fluorescent cell was selected and the mean fluorescence intensity/area for the region was determined and the average values of 15 different regions are presented in Table 1. To quantify the co-localization of fluorescent probes (curcumin and DAPI), images acquired separately for each of the probe were merged using the software.

Results

Uptake measurements

Cellular uptake of curcumin by the three cell lines

Table 1: Cellular uptake, fluorescence spectral properties and intensity variations of curcumin in different cells

Cell lines	λ_{fl}, nm (λ_{ex}= 420 nm)	Cellular uptake (pmoles/ 10^6 cells)	Fl. Intensity/ pmole of curcumin uptake (λ_{ex} = 420 nm)	Confocal imaging data Fl. Intensity / area of cell (λ_{ex} = 458 nm)		
				Control	Treated	Difference
Spleen lymphocytes	504	23.2 ± 4.3	0.023	69 ± 12	125 ± 35	56 ± 35
EL4	500	34.5 ± 6.4	0.064	106 ± 8	212 ± 23	106 ± 23
NIH3T3	535	22.6 ± 3.5	0.007	220 ± 27	259 ± 60	39 ± 5
MCF7	520	44.2 ± 4.5	0.067	208 ± 66	382 ± 91	174 ± 91

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(EL4, NIH3T3 and MCF7) and spleen lymphocytes was calculated at different curcumin treatment concentrations of 10 and 20 nmole/million/ml. After normalization to one nmoles/ ml/million cells of curcumin treatment, cellular uptake was found to vary in the range of 20-40 p moles/million cells for different cells and the actual values are listed in table 1. It can be seen that the tumor cells (EL4 and MCF7) showed higher uptake as compared to normal cells (NIH3T3 and lymphocytes).

Fluorescence spectroscopic studies of intracellular curcumin

Fig.1 gives the fluorescence spectra of curcumin-loaded cells after excitation at 420 nm, along with the normalised fluorescence spectra given as inset of Fig.1. The fluorescence spectrum of curcumin in lymphocytes and EL4 cells is sharp and the maximum is at 500 to 505 nm, while in MCF7 and NIH3T3 cells, the fluorescence spectrum is broad with maximum at 520 to 535 nm. The fluorescence intensity, when compared in these two sets of cells, showed significantly higher intensity in the two tumor cell lines. One of the reasons for such increase in fluorescence could be its increased uptake. To verify this, we calculated the fluorescence intensity to the same level of uptake and compared the fluorescence per unit uptake and the normalized results are listed in Table 1. It is clear that the fluorescence is at least 3-8 times more intense in the tumor cells than the normal cells. The shift in fluorescence spectra and intensity variations clearly indicate that curcumin experiences different hydrophobic environments inside these tumor cells and interacts with the cells differently.

Fluorescence microscopy studies

The above observed fluorescence changes, prompted us to understand its intracellular localization, by fluorescence imaging after exciting intracellular curcumin at 458 nm. Although, the absorption maximum of curcumin is at ~ 420 nm, at 458 nm, it has considerable absorption (45 to 70 % of the maximum absorption in different cells). Figs. 2A and 2B give the confocal micrographs of lymphocytes and EL4 respectively and Figs. 3A and 3B for NIH3T3 and MCF7 cell lines along with their corresponding phase contrast images and a few magnified images. From these images, the average fluorescence intensity per unit area was calculated and the results are listed in Table 1.

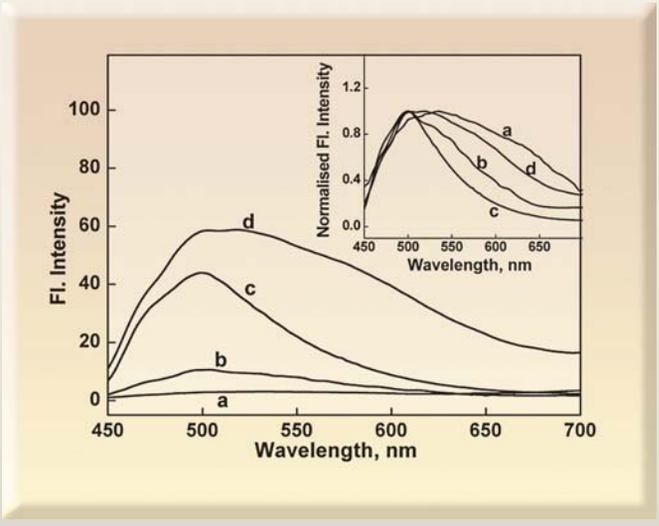

Fig. 1: Fluorescence spectra of curcumin in different cells: (a) NIH3T3, (b) spleen lymphocytes, (c) EL4 and (d) MCF7 after treating with aqueous-DMSO solution of curcumin at concentration of 20 nmoles/million cells/ml. The excitation wavelength was 420nm. Inset shows normalized fluorescence spectra to clearly indicate variation in I^{fl}_{max} for different cells

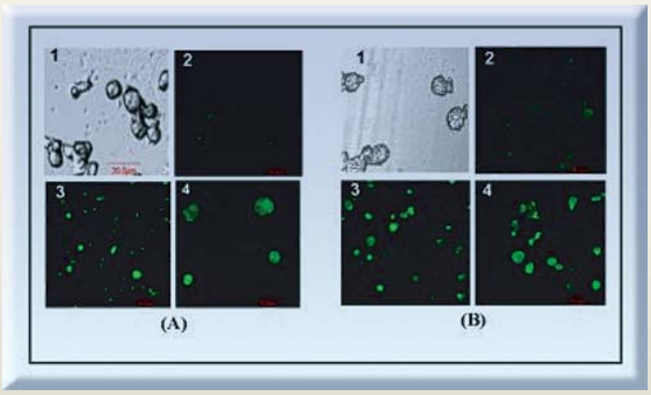

Fig. 2 : Confocal micrographs of cells after treating with curcumin at concentration of 10 nmoles/million cells/ml. (A) Spleen lymphocytes (20X Objective); 1- Phase contrast image, 2-Cells without curcumin treatment, 3- Cells treated with curcumin (2X zoom), 4- Cells treated with curcumin (6X zoom). (B) EL4 cells; 1- Phase contrast image, 2-Cells without curcumin treatment, 3- Cells treated with curcumin (2X zoom), 4- Cells treated with curcumin (3X zoom) Excitation wavelength 458 nm.

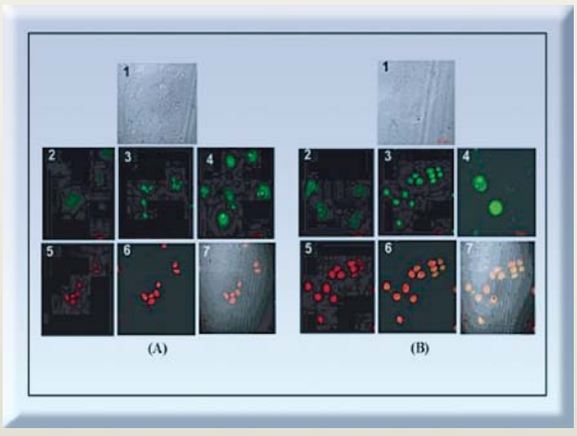

Fig. 3 : Confocal micrographs of cells after treating with curcumin at concentration of 10 nmoles/million cells/ml. (A) NIH3T3 cells (60X objective); 1- Phase contrast image, 2-Cells without curcumin treatment, 3- cells treated with curcumin (1X zoom), 4- Cells treated with curcumin (2X zoom), 5 Cells stained with DAPI (1X zoom), 6 –Images 4 and 5 merged together (1X zoom), 7- Image 4 and 5 merged with image 1 (1X zoom). (B) MCF7 cells (60X objective); 1- Phase contrast image, 2-Cells without curcumin treatment, 3- Cells treated with curcumin (1X zoom), 4- Cells treated with curcumin (2X zoom), 5 Cells stained with DAPI (1X zoom), 6 –images 4 and 5 merged together (1X zoom), 7- image 4 and 5 merged with image 1 (1X zoom). For Curcumin, λ_{ex}– 458 nm and DAPI, λ_{ex}–720 nm, two photon

The results supported our earlier observation that curcumin exhibits more fluorescence in both EL4 and MCF7 cells. Curcumin, being a lipophilic molecule is expected to be localized in the membrane. However, the images of EL4 and lymphocytes show fluorescence emission from the entire cells. Since in these cells, majority of the cell volume is occupied by the nucleus with very little cytoplasm, the emission could be from both the membrane and the nucleus.

In case of MCF7 and NIH3T3 cells, due to their distinct morphology, fluorescence images clearly indicate selective localization in the cell membrane and the nucleus. To further confirm its nuclear localization, these cells were stained with DAPI, a DNA selective fluorescent probe [4]. Fluorescence images of DAPI stained cells were excited using infrared laser tuned to 720 nm and the fluorescent emissions in the range 400-470 were collected. The cells were then subjected to dual staining for which cells treated with curcumin were thoroughly washed, stained with DAPI, and after fixation the images were recorded. The fluorescence from curcumin was pseudo-colored as green and that from DAPI as red

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(Image 5, & 6 of Figs. 3A and 3B). The two images were superimposed as shown in image 7 of Figs. 3A and 3B respectively for NIH3T3 and MCF7 cells. The images clearly showed green and red areas overlapping, confirming the co-localization of curcumin and DAPI in the nucleus.

Discussion

In the recent past, there was extensive research on curcumin and several reports on antioxidant, anti-tumor and chemopreventive activity and preclinical studies, have appeared in literature. However, there are very few papers addressing its cellular uptake and localization. In this paper, applying absorption and unique fluorescence spectral properties of curcumin, we made an attempt to measure quantitative uptake and intracellular localisation of curcumin in four different cell types.

Our results clearly support the earlier reported observation, that tumor cells show preferential uptake of curcumin as compared to normal cells [2]. The fluorescence spectra of cellular curcumin show two interesting factors. The fluorescence intensity is always higher in tumor cells as compared to normal cells and the fluorescence maximum of curcumin in suspension cells is more blue shifted as compared to that in adherent cells. Several recent reports indicate that the fluorescence maximum and fluorescence quantum yield of curcumin are highly sensitive to the medium polarity and availability of the hydrophobic pockets [5,6]. Therefore, the present observations clearly indicate that curcumin interacts differently with different cells. Our preliminary fluorescence imaging studies are very encouraging and clearly show localization of curcumin inside the cells. In all the cells, curcumin fluorescence could be seen in the membrane but in MCF7 and NIH3T3 cells, due to their distinct morphology, localization inside the nucleus is also observed. This is a new and unexpected observation, which needs to be addressed in future.

In conclusion, our present study provides a method to estimate quantitative uptake of curcumin and the results provide confirmation, that tumor cells preferentially take up more curcumin. The fluorescence spectrum of curcumin in tumor cells is easy to detect and can be used as a marker to understand its interaction with different cellular proteins. The fluorescence imaging studies show localization in the cells and a surprising observation of its nuclear localization, this provides a chance to explore new avenues of research to understand its interactions with different nuclear factors and target proteins.

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