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Chlorin p6 preferentially localizes in endoplasmic reticulum and Golgi apparatus and inhibits Ca^{2+} release from intracellular store

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ABSTRACT

Subcellular localization of chlorin p6 in human cerebral glioma (U-87MG) cells was studied using laser scanning confocal microscopy. Localization in sub cellular organelles was ascertained by double labeling with specific fluorescent markers of subcellular organelles. The results reveal that chlorin p6 binds to multiple cellular sites but preferential binding sites are endoplasmic reticulum and Golgi apparatus and it does not bind to mitochondria. Significantly the drug localization pattern of proliferating and differentiated cells was notably distinct. In proliferating cells the internalization of drug was faster than in differentiated cells. Localization of chlorin p6 into the cells inhibited Ca^{2+} release from endoplasmic reticulum and deregulated cellular Ca^{2+} signalling. These results suggest that the fluorescence imaging pattern of chlorin p6 could be useful in identifying the proliferating and differentiated population of cells in tumor tissue.

1. Introduction

Photosensitizing property of various compounds is being exploited in the development of photodiagnostic and phototherapeutic tools [1–9]. These techniques are based on the selective retention of photosensitizing drugs by tumors, viruses and bacteria followed by laser irradiation of appropriate wavelength. When exposed to light in presence of oxygen, the cell bound photosensitizer produces free radicals/singlet oxygen resulting in necrotic and/or apoptotic cell death. Additionally, the fluorescence emitted by the photosensitizer can be utilized for the diagnosis of the disease. Most of the earlier photosensitizers were porphyrin based compounds and they have been studied widely both in the laboratory studies as well as in clinical trials [10–20]. These photosensitizers have shown promising results in the laboratory studies, but their clinical potential was limited due to certain drawbacks such as chemical impurity, less absorption in the red region and skin toxicity. Therefore, variety of second generation photosensitizers are being developed [21–29,31–33].

Photosensitizing drug chlorin p6 is a chlorophyll derivative and is gaining considerable interest in recent years [21–29]. In view of higher absorption in the red region, excellent tumor localizing property and high photosensitizing efficacy several analogues of chlorins are being synthesized and their photophysical, photochemical and photosensitizing properties in solutions and in cultured cells are being examined. Postogo et al. have studied the photosensitization of skin fibroblasts and HeLa cells by chlorin derivatives [22]. Feofanov et al. compared the photodynamic properties of various cycloimide derivatives of chlorin p6 [23] and showed that the intracellular uptake and photosensitivity was directly dependent on the substituent groups. The drug uptake by cells was found to be dependent on the structure of chlorin p6 and the nature of liposomes employed for delivering the chlorin p6. Pogue et al. have reported that the subcellular localization and rate of photobleaching are crucial in photocytotoxicity of ovarian cancer cells by two structurally similar chlorin p6 photosensitizers [24]. Kelbauskas and Dietel have studied the internalization of chlorin photosensitizers in A431 human endothelial carcinoma cells using single and two-photon fluorescence imaging [25]. They have demonstrated that the cells internalize the photosensitizer aggregates by endocytosis and its monomerization inside the cells. Sharma et al. studied the influence of extracellular pH on the uptake, photosensitization of chlorin p6 and mode of cell death in human colon and breast adenocarcinoma cells [26–28]. They found that the both drug uptake and mode of cell death by necrosis or apoptosis are highly dependent on the extracellular pH. Dube et al. from the same group have examined the photodynamic action of chlorin p6 using hamster cheek pouch model [29].

For efficient photodynamic action and for the development of fluorescent-based diagnostic and therapeutic techniques, it is important to know the sites of cellular localization of photosensitizing drug. Fluorescence is a very sensitive and powerful technique to investigate the real time molecular events in intact

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single living cell [30]. The cellular localization of some photosensitizing drugs using fluorescence imaging has been studied in some types of cells [31–33]. So far, such studies have not been performed for chlorin p6 in any type of cells. In the present paper, we report the cellular localization of chlorin p6 in human cerebral glioma (U-87MG) cells. The internalization of drug was faster in early phase/proliferating cells whereas it was considerably slower in the late phase/differentiated cells. We have demonstrated that chlorin p6 localizes preferentially in endoplasmic reticulum and inhibits Ca^{2+} release from intracellular pools.

2. Materials and methods

2.1. Materials

Eagle's minimal essential medium (EMEM), neomycin sulphate, thapsigargin, cyclopiazonic acid, sodium pyruvate and N-2-hydroxyethylpiperzaine-N′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Aldrich Foreign Holding Co., Bangalore (India). Fura-2 acetoxymethyl ester (Fura-2AM), pluronic, Golgi marker, ER Tracker, mitotracker-green and lysotracker red were procured from Molecular Probes, Eugene, OR, USA. Fetal calf serum was from GIBCO-BRL India. Chlorin p6 was prepared as described earlier with minor modifications [26]. Stock solution of chlorin p6 was prepared in HEPES-buffered salt solution (HBSS). All other chemicals, obtained from commercial sources, were of analytical grade.

2.2. Cell culture

U-87 MG cells were obtained from National Centre for Cell Sciences (NCCS), Pune, India and were cultured as described earlier [14]. Cells were cultured on poly-L-lysine coated glass coverslips in EMEM containing 1 mM sodium pyruvate, 10% FCS, 2.2 mg/L HEPES, 50,000 units/L benzyl penicillin, 3500 units/L streptomycin and 2.2 mg/L nystatin and were maintained at 37 °C in an atmosphere of 5%CO_2.

Proliferating cells were obtained by mitotic shake off [14]. Cells were plated in plastic culture flasks and allowed to grow for 24 h. The loosely attached cells were removed by shaking the flasks in rotary shaker and the cells detached in the first shake off were discarded. The attached cells were replenished with pre-warmed medium and incubated for 40 min at 37 °C. The shaking procedure was repeated and the mitotic cells were collected from the medium by centrifugation. Subsequently, the cells were seeded on poly-L-lysine coated glass cover slips. These cells were allowed to attach the cover slip for 2–3 h. Differentiated cells were obtained by incubating the cells with reduced serum containing media [15]. The cells were grown in normal growth medium for 48 h, subsequently, the cells were deprived of serum (from 10% to 0.5%) and grown further, and the experiments were performed after 96 h.

2.3. Laser scanning confocal microscopy

Subcellular localization of chlorin p6 was studied using Olympus FV1000 confocal microscope with 60X water immersion apo objective. Cells grown on the coverslips, were washed with HBSS and coverslip was mounted on the perfusion chamber and the same was placed on the stage of microscope. Chlorin p6 (5 μM) was added to the static bath and fluorescence images were captured after 5, 10 and 30 min. Air cooled argon ion laser at 515 nm was used to excite chlorin p6.

The localization of chlorin p6 in subcellular organelles was visualized by labeling the cells with fluorescent probes specific to the organelle markers such as Golgi marker, lysotracker, mitotracker and ER tracker. Golgi apparatus, mitochondria and lysosomes were visualized by staining the cells with 2 μg/mL BODIPY TR® ceramide, 250 nM mitotracker green and 50 nM lysotracker red, respectively, for 30 min. Cells loaded with organelle markers were washed with HBSS and the cover slip was mounted on perfusion chamber and placed on microscope stage as mentioned above. A Helium–Neon laser was used as excitation source at 543 nm for lysotracker and Golgi marker, whereas, air cooled argon ion laser at 488 nm was used to excite ER tracker and mitotracker.

Co-localization of the organelle markers with chlorin p6 was further analyzed by constructing the topographic profiles. The topographic profiles of chlorin p6 and corresponding organelle tracker in single cells were recorded along a line within the cell and the extent of overlap between the two was analyzed using the Fluoview software (Olympus). Fluorescence spectra of organelle probes and chlorin p6 loaded cells were measured using laser scanning confocal microscope and were analyzed using Fluoview software.

2.4. $[Ca^{2+}]_i$ imaging

The Ca^{2+} sensitive fluorophore Fura-2 was used for measuring $[Ca^{2+}]_i$. All fluorescence measurements were made from sub confluent areas of coverslips as described earlier [34]. Briefly, U-87MG cells were incubated with 2.5 μM Fura-2AM in HBSS containing 0.08% (w/v) pluronic acid for 30 min at 37 °C. Cells were then washed with medium and allowed to de-esterify for 30 min before initiation of the experiments. Cover slip with Fura-2 loaded cells was mounted on the recording chamber and placed on the stage of an inverted microscope (Olympus IX70, Japan). Real time $[Ca^{2+}]_i$ imaging was performed with a fluorescence imaging system (TILL Photonics, Germany). The cells were alternately excited at 340 and 380 nm with xenon arc lamp source coupled to a high-speed monochromator. The emitted fluorescence was selected with filter and images were acquired with a 12-bit peltier cooled CCD camera. Fields containing 20–30 cells were selected for imaging. The data acquisition and analysis was done with TILL Vision software. For calcium imaging experiments, the basal level of $[Ca^{2+}]_i$ was measured in Ca^{2+} free extra cellular solution containing 1 mM EGTA and required amount of calcium was added to the static bath 50 s after starting the recording.

3. Results and discussion

3.1. Cellular distribution of chlorin p6 in different phases of growth

The subcellular localization of chlorin p6 in U-87MG cells in early phase (24 h in vitro) and late phase (96 h in vitro) phase of growth of U-87MG cells was studied. In case of cells in the early phase of growth, chlorin p6 was initially localized on the cell membrane and migrated into the cytoplasm after about 5 min of incubation (Fig. 1, left panel). Incubation for longer time (30 min) resulted in intracellular staining with an intense fluorescence in the perinuclear area. Cells in the late phase of growth also showed initial staining of chlorin p6 on the cell membrane (Fig. 1, right panel) but the migration of drug into the cytoplasm was slower as the drug remained in the membrane for more than 10 min. After 30 min of incubation, a clear cytoplasmic staining was observed again mostly in the perinuclear region and also, the morphology of the cells was totally altered, as these cells appeared round. On the other hand, the cells in the early phase of growth did not show any morphological change even after incubation with chlorin p6 for 45 min (data not shown). The intense fluorescence in perinuclear region suggests the localization of chlorin p6 in ER and Golgi apparatus.