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Chemical principles for a novel fluorescent probe with high cancer-targeting selectivity and sensitivity

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Abstract

Understanding of principles governing selective and sensitive cancer targeting is critical for development of chemicals in cancer diagnostics and treatments. We determined the underlying mechanisms of how a novel fluorescent small organic molecule, 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC), selectively labels cancer cells but not normal cells. We show that BMVC is retained in the lysosomes of normal cells. In cancer cells, BMVC escapes lysosomal retention and localizes to the mitochondrial or to the nucleus, where DNA-binding dramatically increases BMVC fluorescence intensity, allowing it to light up only cancer cells. Structure-function analyses of BMVC derivatives show that hydrogen-bonding capacity is a key determinant of lysosomal retention in normal cells, whereas lipophilicity directs these derivatives to the mitochondria or the nucleus in cancer cells. In addition, drug-resistant cancer cells preferentially retain BMVC in their lysosomes than drug-sensitive cancer cells, and BMVC can be released from drug-resistant lysosomes with lysosomotropic agents. Our results further our understanding of how properties of cellular organelles differ between normal and cancer cells, which can be exploited for diagnostic and/or therapeutic use. We also provide physiochemical design principles for selective targeting of small molecules to different organelles. Moreover, our results suggest that agents which can increase lysosomal membrane permeability may re-sensitize drug-resistant cancer cells to chemotherapeutic agents.

Introduction

Despite substantial progress in understanding the fundamental mechanisms of carcinogenesis, cancer remains one of the leading causes of death worldwide. Innovative non-invasive methods for early diagnosis as well as targeted therapeutic approaches for many types of cancer are urgently needed. To achieve efficacy and accuracy, cancer diagnostics and treatments must exhibit exquisite specificity and sensitivity to selectively detect and target cancer cells, especially considering that cancer cells are vastly outnumbered by normal cells in patients.

We have previously described the small molecule 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC), engineered to bind DNA, whose fluorescence quantum yield increases 100-fold upon binding DNA¹. Interestingly, we found that after incubation with BMVC, strong fluorescent signals could be detected in the nucleus of multiple human cancer cell lines but not corresponding normal cells. Based on these findings, we applied BMVC to clinical diagnosis of malignant neck lumps and showed that the positive predictive value (PPV) of the BMVC test is approximately 70%, whereas the negative predictive value (NPV) of this method is approximately 90%². Despite this exciting success, the underlying mechanism of how BMVC differentiates cancer cells from normal cells remains unclear. This information will further our understanding of mechanisms that control specific targeting of cancer cells and will aid in the design of potential new agents for early cancer detection.

Lysosomes, first described by de Duve in 1955³, play an important role in intracellular degradation of endogenous and exogenous macromolecules. Because exogenous drugs often enter the lysosomal compartment via endocytosis, lysosomes have emerged as a major target for drug delivery⁴. Recent studies demonstrate that the properties of lysosomes differ in normal and cancer cells⁵. For example, the lysosomal pH is often higher in cancer than in normal cells⁶, and expression of lysosomal cathepsins increases with cancer progression and invasion⁷. In addition, lysosomal membrane permeability is perturbed in cancer cells. Oxidative stress⁸, Ras activation⁹, TNF- α ¹⁰, and lysosomotropic detergents¹¹ induce lysosomal membrane permeabilization, release of cathepsins into the cytoplasm and subsequent cell death¹². Oncogenically-transformed and immortalized mouse embryonic fibroblasts (MEFs) are much more sensitive to TNF-mediated, cathepsin-dependent cell death than wild-type MEFs¹³. Hsp70, which inhibits lysosomal membrane permeabilization, is upregulated in several types of primary tumors¹⁴, and depletion of Hsp70 triggers cathepsin-mediated cell death in tumor cell lines¹⁵. At present, it is not understood what chemical and/or physical properties determine how a molecule partitions between the lysosome and cytoplasm in different cells. Although not yet demonstrated, it may be possible to exploit the differential permeability of lysosomes in cancer and normal cells for cancer diagnostic and therapy.

In this study we determine the mechanism underlying BMVC's cancer targeting specificity. We show that BMVC enters and is retained in the lysosomes of normal cells, whereas in cancer cells, BMVC escapes from lysosomes and localizes to the mitochondria or to the nucleus, where it binds to DNA and shows hyperfluorescence. From a panel of BMVC

derivatives, we show that hydrogen bonding capacity is a major determinant of lysosomal retention in normal cells, and lipophilicity governs the preferential localization of BMVC derivatives to the mitochondria over the nucleus in cancer cells. Finally, we show that drug-resistant cancer cells exhibit increased lysosomal BMVC retention relative to drug-sensitive cancer cells, and that this can be reversed by treatment with lysosomotropic agents. Our study presents proof-of-principle data for exploiting differences in subcellular localization for cancer targeting for both diagnosis and treatment strategies.

Results

Subcellular localization of BMVC in cancer cells versus normal cells

We first tested the possibility that BMVC enters cells by diffusion across the plasma membrane, and somehow this diffusion is different between normal and cancer cells. We incubated cells with BMVC at 4°C, a condition where energy-dependent mechanisms are inhibited. BMVC fluorescence was not detected in both normal and cancer cells under this condition. In contrast, Hoechst 33342, a membrane-permeable DNA dye, was readily detected in the cells and a closely related but membrane-impermeable DNA dye, Hoechst 33258, was not detected (Fig. S1). Therefore, BMVC likely enters the cells via energy-dependent mechanisms such as endocytosis, and the contribution of diffusion across the plasma membrane for BMVC uptake is very small. Experiments with knocking down clathrin or caveolin showed that either pathway is not essential for this process, and instead they may function redundantly (data not shown).

To gain insight into why BMVC lights up the nuclei of cancer cells but not normal cells^{1b, 16}, we examined the subcellular localization of BMVC in human normal lung (MRC-5) cells and human lung cancer (CL1-0) cells. Consistent with previous results^{1b, 16}, a strong fluorescent signal was observed in the nuclei of BMVC-treated CL1-0 cells, whereas no fluorescence was observed in the nuclei of BMVC-treated MRC-5 cells (Fig. 1A). The fluorescence intensity difference of BMVC between cancer and normal cells was quantitatively measured by flow cytometry (Fig. 1B). The fluorescent signal in the cancer cell nuclei was dramatically reduced upon treatment with DNase and was moderately reduced upon treatment with RNase (Fig. 1C). These results are consistent with the fact that the fluorescence quantum yield increases dramatically when BMVC binds DNA and/or RNA^{1a}. In addition, we detected weak BMVC fluorescence in vesicular structures in normal MRC-5 cells, which colocalized with LysoTracker red but not MitoTracker red staining (Fig. 1D). By contrast, BMVC fluorescence partially colocalized with MitoTracker red, but not LysoTracker red staining in CL1-0 cells. These results indicate that BMVC is retained in the lysosomes and is excluded from the nuclei of normal cells, which could explain the observed selective hyperfluorescence of BMVC in cancer cell nuclei.

Lysosomal retention modulates the localization of BMVC in normal cells

The above results suggest that lysosomal retention prevents BMVC from gaining access to the nuclei and mitochondria in normal cells. To test this possibility, we micro-injected BMVC directly into the cytoplasm of primary human normal foreskin fibroblast cell line (BJ) to bypass the transport of BMVC to lysosomes via endocytosis. As shown in Fig. 2A,

BMVC rapidly accumulated in the nuclei of the injected cells, indicating that once outside the lysosome, BMVC can freely access the nucleus of normal cells. In contrast, the co-injected Texas Red labeled 70-kDa dextran, which cannot pass through nuclear pores, remained in the cytoplasm. Next, fixed and permeabilized MRC-5 cells, in which the lysosomal membrane integrity was impaired, were incubated with BMVC. As shown in Fig. 2B, strong BMVC fluorescence similar to that in CL1-0 cells was observed in the fixed and permeabilized nuclei of MRC-5 cells. This result was confirmed by flow cytometric analysis of BMVC fluorescent intensity of MRC-5 and CL1-0 cells (data not shown).

To confirm that lysosomal membrane permeability regulates the retention of BMVC in lysosomes, we used L-leucyl-L-leucine methyl ester (LeuLeuOMe), a lysosomotropic agent that is converted to a membranolytic compound upon endocytosis resulting in cathepsin release from lysosomes and cell death¹⁷. We confirmed that LeuLeuOMe increases lysosomal membrane permeabilization in MRC-5 and CL1-0 cells and causes cytotoxicity above 1 mM (Fig. S2). Used at subtoxic level of LeuLeuOMe (0.5mM), Fig. 3A shows that BMVC fluorescence can be detected in the nucleus of MRC-5 cells, which correlated with an increase in the fluorescence intensity in these cells (Fig. 3C). By contrast, LeuLeuOMe treatment only caused a small increase in BMVC fluorescence in CL1-0 cells (Fig. 3B and 3C). These results demonstrate that BMVC is actively retained in the lysosomes in normal cells and that it can be rapidly released when lysosomal membrane integrity is disrupted.

Hydrogen bonding capacity affects lysosomal retention of BMVC derivatives

We then characterized the structural and physiochemical properties that contribute to the retention of BMVC in the lysosomes of normal cells. Hydrogen bonding capacity (HBC) is one of the properties of molecules that inversely correlates with permeance and oil-water partition coefficient^{4, 18}. Based on their calculation, HBC is 11 of the compound with one charged nitrogen while the HBC is 22 of the compound with two charged nitrogens. Compounds with an HBC 18 have been shown not to cross the lysosomal membrane in normal cells⁴. Consistently, the predicted HBC is 22 for BMVC with two 1-methyl-4-vinylpyridinium cations. This indicates that BMVC derivatives with higher HBC might be more strongly retained by the lysosomes, whereas BMVC derivatives with lower HBC might not be retained by the lysosomes, instead being released and subsequently translocating to other cellular compartments, such as nucleus and mitochondria. To test this hypothesis, we synthesized a number of BMVC derivatives with different HBC and measured their lysosomal retention in cells. As predicted, the monocation BrMVC, which has a low HBC (HBC=11) co-localized poorly with the LysoTracker red stain, indicating that BrMVC likely partitions into both the cytoplasmic and lysosomal compartments in MRC-5 cells (Fig. 4A). By contrast, the trication BMVC-8C (HBC=33) and the tetracation (BMVC)₂-8C (HBC=44), which have much higher HBCs, co-localized strongly with the LysoTracker red stain. The percentage of BMVC derivatives on lysosome (lysosome overlay ratio %) was quantified by analyzing about 100 cells in at least three independent experiments by Metamorph 7.6 (Fig. 4B). Because BrMVC, which is not retained well by the lysosome, is also smaller in size than BMVC-8C and (BMVC)₂-8C, we would like to rule out that molecular size of the compound also contributes to our results. We compared the lysosomal retention of (BMVC)₂-8C (HBC=44) and (MVC)₂-8C (HBC=22), which have

comparable molecular size, but different HBCs. As predicted, (MVC)₂-8C is retained in the lysosomes of MRC-5 to a similar extent to BMVC (HBC=22) but is less strongly retained in the lysosomes of MRC-5 cells than (BMVC)₂-8C (HBC=44) (Fig. 4C). These results demonstrate that HBC is a major determinant of lysosomal retention of BMVC in normal cells. We also tested if BMVC derivatives with more positive charged nitrogen would be retained in cancer cells. We found that BMVC-8C (HBC=33) escaped lysosomal retention while (BMVC)₂-8C (HBC=44), which contains four cations, partially colocalized with LysoTracker blue in CL1-0 cancer cells (Fig. S3). Accordingly, HBC threshold for crossing the lysosomal membrane is different between cancer cells relative to normal cells.

Lipophilicity facilitates the mitochondrial localization of BMVC derivatives

We next sought to identify factors that affect nuclear versus mitochondrial localization of BMVC in cancer cells. In BMVC-treated CL1-0 cells, strong fluorescence was detected in the nuclei and weak fluorescence was detected in the mitochondria, suggesting that BMVC enters and binds to DNA in both compartments upon lysosomal release. We show that (MVC)₂-8C localizes to the mitochondria to a greater extent than the nuclei, whereas BMVC was found to localize to the nuclei to a greater extent than the mitochondria in CL1-0 cells (Fig. 1D). Murphy *et al.*¹⁹ suggested that cationic, lipophilic compounds tend to target and accumulate in mitochondria due to the negative mitochondrial membrane potential. Recently, Horton *et al.*²⁰ also demonstrated that lipophilicity facilitates the mitochondrial localization of synthetic cationic peptides, whereas hydrophilic amino acids have a neutral effect on the distribution of peptides to the nuclei or mitochondria. We examined a series of BMVC derivatives with different lipophilicities and hydrophilicities in cancer cells to dictate localization of these compounds to the mitochondria or to the nucleus following lysosomal release.

BMVC derivatives were synthesized in which either the lipophilicity or the hydrophilicity were increased by substituting a variable length alkyl linker and an N-methyl-piperidinium cation at the N-9 position of BMVC, respectively. The lipophilicity of each BMVC derivative was calculated from the logarithm of the octanol/water partition coefficient (log P), and their subcellular localization was determined using confocal fluorescence microscopy. As shown in Fig. 5A and Fig. S4, BMVC derivatives with relatively high lipophilicities, such as BMVC-9C and BMVC-12C, localized primarily to the mitochondria of cancer cells, whereas BMVC derivatives with relatively low lipophilicities, such as BMVC-4C, localized preferentially to the nuclei rather than to the mitochondria. Consistent with these results, BMVC-8C3O, in which the C12 chain of BMVC-12C was substituted with hydrophilic tetraethylene glycol and has lower lipophilicity, localized to both the nucleus and the mitochondria of CL1-0 cells, whereas BMVC-12C localized primarily to the mitochondria (Fig. 5B). The correlation between mitochondrial localization and lipophilicity of the same panel of BMVC derivatives was also demonstrated in Hela cervical and MCF-7 breast cancer cell lines (Fig. S5).

Fig. 5C summarizes the effect of lipophilicity on mitochondrial localization for the BMVC derivatives. BMVC derivatives with three positive charges and low lipophilicity (log P < -2.15) localized primarily to the nucleus, whereas BMVC derivatives with higher

lipophilicity ($\log P > -2.0$) localized primarily to the mitochondria and were excluded from the nuclei. These data confirm that lipophilicity facilitates mitochondrial localization of BMVC derivatives in CL1-0 cells.

Subcellular differences between drug-sensitive and drug resistant cancer cells

We next examined BMVC fluorescence in drug sensitive human breast cancer cells (MCF-7) and its Adriamycin resistant pair (MCF-7/ADR). We confirmed this drug sensitive and multidrug resistant pair by measuring the cytotoxicity (IC_{50}) of the chemotherapeutic drug doxorubicin in MCF-7 ($IC_{50}=0.625 \mu M$) and MCF-7/ADR ($IC_{50}=20 \mu M$) cells (data not shown). Consistent with our previous results in CL1-0 cells, BMVC was released from the lysosomes and entered the nucleus of MCF-7 cells. By contrast, BMVC localized to the lysosomes of MCF-7/ADR cells (Fig. 6A and 6B), similar to the localization of BMVC observed in normal cells.

To elucidate the underlying mechanism for lysosomal retention of BMVC in MCF-7/ADR cells, we used verapamil, an L-type calcium channel blocker, to inhibit the multi-drug efflux pump P-glycoprotein or LeuLeuOMe to increase the lysosomal membrane permeability. Upon addition of $50 \mu M$ verapamil, the fluorescence of doxorubicin increased twofold without affecting BMVC fluorescence. By contrast, addition of $0.5 mM$ LeuLeuOMe resulted in increased BMVC fluorescence (more than tenfold) with no difference in doxorubicin fluorescence (Fig. 6C). The increase of BMVC fluorescence in MCF-7/ADR cells upon LeuLeuOMe treatment was associated with increased nuclear BMVC fluorescence (Fig. 6D).

We also used BMVC derivatives to examine the effect of HBC on the lysosomal retention of BMVC derivatives in MCF-7/ADR cells. Consistent with our previous findings in normal cells, BMVC (HBC=22) and BMVC-8C (HBC=33) were retained in the lysosomes, whereas BrMVC, which harbored the lowest HBC (HBC=11), escaped from the lysosomes and entered the nuclei of MCF-7/ADR cells (Fig. S6). Together, our results show that lysosomal membrane permeability is reduced in drug-resistant cancer cells, and that this difference can be readily detected by BMVC.

Discussion

In this study, we determined the mechanisms underlying how BMVC differentiates between cancer and normal cells. We show that subcellular localization of BMVC is essential for its ability to light up cancer but not normal cells. In particular, the ability to retain BMVC in the lysosome is the key determinant. Treatment of normal cells with lysosomotropic agent LeuLeuOMe or direct microinjection of BMVC in the cytoplasm allowed BMVC to gain access to the mitochondria or to the nucleus, where it binds DNA and emit strong fluorescence. NH_4Cl and chloroquine treatment which raise the internal pH of the lysosomes²¹ also increased nuclear fluorescence of BMVC in normal cells (Fig. S7). However, the effect of LeuLeuOMe is much bigger than that of pH-raising drugs.

Our results are consistent with the notion that lysosomal membrane permeability is increased in cancer cells. Cancer-associated changes in lysosomal membrane permeability

had been linked with both tumorigenesis and metastasis. Increased levels of lysosomal cathepsins caused by changes in the lysosomal membrane permeability have been associated with cancer progression and invasion^{7a, 10, 22}. It was also shown that heat shock protein 70 (Hsp70) is frequently up-regulated in tumors¹⁴, and that depletion of Hsp70 in breast, pancreatic, or colon cancer cells leads to increased lysosomal membrane permeability and cathepsin-mediated cell death without any external death stimuli^{15, 23}. The difference in lysosomal membrane permeability between cancer cells and normal cells may thus be exploited for selective cancer diagnostic and therapeutics. To this end, our characterization of BMVC derivatives with different chemical properties establishes new molecular principles for the design of selective cancer targeting reagents. Our results showed that HBC is a key determinant in lysosomal retention. Compounds with HBC 18⁴ can be retained in the lysosome of normal cells, whereas HBC<44 are likely released from the lysosome of cancer cells (Fig. 4 and Fig. S3). Accordingly, compounds with HBC larger than 18 and smaller than 44 may selectively label cancer cells.

After release from the lysosome, the localization of BMVC and its derivatives to the nucleus or mitochondria is mainly determined by lipophilicity. BMVC derivatives with higher lipophilicity preferentially accumulate in the mitochondria (Fig. 5 and Fig. S5). Our results show that BMVC-12C, which has a higher lipophilicity than BMVC, is selectively targeted to the mitochondria of CL1-0 cells. These findings demonstrate that small molecules can be selectively targeted to specific subcellular organelles by modifying specific physiochemical properties of the molecules for both cancer diagnosis and treatment.

We envision that BMVC will be best used as an early cancer detector, before cancer cells develop drug-resistance. However, we also show that BMVC can be used to distinguish between drug-sensitive and drug-resistant cancer cells. These findings are consistent with studies showing that melanosomal sequestration of cytotoxic drugs contributes to the relative chemoresistance of malignant melanomas²⁴. In this scenario, because BMVC fluorescence is lower in drug-resistant cancer cells compared to drug-sensitive cancer cells, and may be similar to normal cells, another tumor marker will be used in conjunction to detect cancer cells that have developed drug-resistance. Our results also carry exciting clinical implications for agents that increase the lysosomal membrane permeability, as our data suggest that such drugs might re-sensitize drug-resistant cancer cells to chemotherapeutic agents.

In summary, we combined methods from chemical and cell biological disciplines to determine chemical principles for specific targeting to intracellular organelles. These principles enable a fluorescent small molecule, BMVC, to selectively and sensitively target cancer cells but not normal cells. To our knowledge, this is the first example to systematically engineer a fluorescent tumor marker by selective targeting of intracellular organelles. Information gained from our study also furthers our understanding of how properties of cellular organelles differ between normal and cancer cells, which can be exploited for diagnostic and/or therapeutic use.

Materials and Methods

Chemicals

L-leucyl-L-leucine methyl ester (LeuLeuOMe) was purchased from BaChem. The synthesis of BMVC was previously described^{1a} and the synthesis of BMVC derivatives can be found in Supplementary. Fig. 7 shows the chemical structures of the BMVC derivatives used in this study. Chloroquine, verapamil, acridine orange (AO), and ammonium chloride (NH₄Cl) were purchased from Sigma.

Lipophilicity

The lipophilicity of each BMVC derivative was determined from the logarithm of the *n*-octanol/water partition coefficient as previously described²⁵. Briefly, a mixture of dd-H₂O and *n*-octanol was prepared and incubated overnight before use. BMVC derivatives were added to the dd-H₂O phase at 500 μM and then mixed with an equal volume of *n*-octanol. Absorbance in the dd-H₂O (*A_w*) and *n*-octanol (*A_o*) phases was measured using an ELISA Reader (Thermo Electron Corporation). Log P was determined using the equation $\log P = \log[A_o/A_w]$.

Cell lines

The MRC-5 human lung fibroblast cell line, the BJ human foreskin fibroblast cell line, and the MCF-7 human breast cancer cell line were purchased from ATCC (American Type Culture Collection). Cells were grown in EMEM supplemented with 10% FBS. The CL1-0 human lung cancer cell line was kindly provided by Prof. C. T. Chen of the National Taiwan University and was grown in RPMI1640 supplemented with 10% FBS. The MCF-7/ADR cell line was kindly provided by Prof. Y. H. Chen of the National Taiwan University and was grown in DMEM supplemented with 10% FBS. All cells were grown in an incubator supplemented with 5% CO₂ at 37°C.

Fluorescence microscopy

CL1-0 or MRC-5 cells were incubated with 1 or 5 μM BMVC or BMVC derivatives for 24 h and then were observed by epi-fluorescence or confocal microscopy. Cells were fixed by incubation in 4% paraformaldehyde, 0.1% Triton X-100 for 10 min. Fixed cells were treated with 0.2 ml of 2 mg • ml⁻¹ RNase or 1 mg • ml⁻¹ DNase at 37°C for 30 min. To investigate the subcellular localization of BMVC, CL1-0 or MRC-5 cells were incubated with 1 μM BMVC or BMVC derivatives for 24 h. Cells were subsequently treated with 40 nM MitoTracker red CMXRos (Invitrogen) for 30 min, 200 nM LysoTracker red DND-99 (Invitrogen) for 10 min, 3 μM LysoTracker blue DND-22 (Invitrogen) for 5 min, or 40 nM Hoechst 33342 (Sigma) for 10 min. Stained cells were washed twice with PBS and were visualized by confocal microscopy. Fluorescence excitation was carried out at 458 nm for BMVC, 532 nm for LysoTracker red and MitoTracker red, and 405 nm for Hoechst 33342 and LysoTracker blue. Co-localization of BMVC and MitoTracker or LysoTracker was calculated as a percentage value for each cell using MetaMorph 7.6 software (Molecular Devices). Values were expressed as the mean ± S.E., including 100 cells from two independent experiments.

Microinjection

BJ primary fibroblasts were grown on glass cover slips, mounted onto a Ludin imaging chamber (Life Imaging Services) and microinjected with 170 μM BMVC and 2.5 mg ml^{-1} Texas Red-conjugated 70-kDa dextran (Invitrogen) at 37°C as previously described²⁶. Injected cells were incubated for 5 min, transferred to fresh CO₂-independent medium (Invitrogen) and visualized on an Axiovert 200 M microscope with a LD Plan-Neofluar 40x/1.3 DIC objective (Zeiss) and an Orca-285 CCD camera (Hamamatsu Photonics). The software package Openlab 4.02 (Improvision) was used to record images of live cells.

Flow cytometry

CL1-0 and MRC-5 cells were incubated with 1 μM BMVC or BMVC derivatives for 5 h. Cells were collected by centrifugation and were resuspended in 300 μl PBS, adjusted to a density of approximately 1×10^6 cells/ml, and analyzed by flow cytometry (BD, FACSCalibur). Ten thousand cells were analyzed in each experiment. The mean fluorescence intensity at 564–606 nm was measured upon excitation at 488 nm. The error bars were calculated based on three independent experiments.

Cell Viability Assay (MTT assay)

Cells were grown in 96-well plates (2,000 per well) in a 5% CO₂ incubator at 37°C. To examine the short-term cytotoxic effect, cells were then incubated with different concentrations of LeuLeuOMe for 24 h. The cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analyzed spectrophotometrically at the absorbance of 570 nm. The error bars were calculated based on three independent experiments.

Endocytosis experiment

The low temperature experiments²⁷ which can prohibit energy dependent process were used here to examine the major pathway for BMVC uptake. Cells were precooled at 4°C for 1 h, then incubated with 1 μM BMVC for 1 h and co-stained with 1 ng ml^{-1} Hoechst 33258 or 1 ng ml^{-1} Hoechst 33342 for 10 min. The cells images were observed by epi-fluorescence microscopy.

Conclusions

In summary, we have demonstrated that cancer-associated changes in lysosomal membrane permeability enable the fluorescent small organic molecule BMVC to selectively label the nucleus of cancer cells but not normal cells. Our structure-function analyses of BMVC derivatives identify critical chemical properties underlying selective targeting to different cellular organelles. Our studies show that differences in lysosomal membrane permeability between cancer cells and normal cells and between drug-sensitive and drug-resistant cancer cells may be exploited for diagnostic or therapeutic purposes. In addition, the use of small fluorescent molecules remains invaluable for the discovery of novel intracellular differences between different cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Cancer is one of the leading causes of death, and 7.6 million people died of cancer each year world wide. Novel diagnostic and therapeutic agents are urgently needed, which requires understanding of principles governing selective and sensitive cancer targeting. In this study, we integrated methods from chemical and cell biological disciplines to determine the underlying mechanisms of how a novel fluorescent small organic molecule, BMVC, selectively labels cancer cells but not normal cells. We uncover how properties of cellular organelles differ between normal and cancer cells, and between drug-resistant and drug-sensitive cancer cells, and show that these differences can be exploited for diagnostic and/or therapeutic use. We also identify chemical principles for specific targeting to intracellular organelles.

Fig. 1. The fluorescent small molecule 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) is localized in the nucleus and mitochondria of cancer cells and in the lysosomes of normal cells

(A) Phase contrast and epi-fluorescence images show strong nuclear fluorescence in BMVC-stained CL1-0 cells (upper panel) and cytosol fluorescence in BMVC-stained normal human fetal lung MRC-5 cells (lower panel) and. (B) Time-dependent fluorescent intensity measured by flow cytometry of 1 μ M BMVC was significantly higher in CL1-0 than in MRC-5 cells. (C) Phase contrast and epi-fluorescence images show moderately or strongly reduced fluorescence in CL1-0 cells treated with RNase (left panel) or DNase (right panel),

respectively. **(D)** Confocal fluorescence images of CL1-0 (left panel) or MRC-5 cells (right panel) show that BMVC co-stains with MitoTracker red in CL1-0 cells and co-stains with LysoTracker red in MRC-5 cells. In all panels, the scale bar is 15 μm .

Fig. 2. Lysosomal retention of BMVC prevents BMVC from gaining access to the nuclei and mitochondria in normal cells

(A) BMVC and Texas Red-labeled dextran were microinjected into the cytoplasm of primary human normal foreskin fibroblasts (BJ) and incubated for 5 min. The incubation medium was changed and live cells were visualized using confocal microscopy. BMVC (green) was concentrated in the nucleus, while Texas Red-labeled dextran (red) was restricted to the cytoplasm. The scale bar is 10 µm. (B) After fixation with 4% formaldehyde and 0.1% Triton X-100, BMVC fluorescence was detected in the nucleus of both CL1-0 (left panel) and MRC-5 cells (right panel). Bright field and confocal fluorescence images are shown. The scale bar is 15 µm.

A **B**

C

Fig. 3. Lysosomal leakage of 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) in MRC-5 and CL1-0 cells after L-leucyl-L-leucine methyl ester (LeuLeuOMe) treatment
 Confocal fluorescent images of MRC-5 (A) and CL1-0 (B) cells incubated with 5 μ M BMVC for 24 h (upper panel) or with 5 μ M BMVC followed by LeuLeuOMe 0.5 mM for 1 h (lower panel). Cells were co-stained with 100 nM Hoechst 33342 for 10 min. A 1% propidium iodide (PI) solution was also added to distinguish living cells from dead cells. The scale bar is 25 μ m. (C) Flow cytometric analysis of the ratio of BMVC fluorescence in 0, 0.5 and 1 mM LeuLeuOMe-treated CL1-0 and MRC-5 cells to evaluate BMVC fluorescence. PI was used in this experiment, and only PI-negative live cells are gated for inclusion in the analysis

Fig. 4. Hydrogen bonding capacity (HBC) regulates lysosomal retention of 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) derivatives in normal lung MRC-5 cells

(A) Confocal fluorescence images of MRC-5 cells incubated with 1 μ M BrMVC (monocation), 5 μ M BMVC (dication), or 10 μ M BMVC-8C (trication) and co-stained with 200 nM LysoTracker red. Because the fluorescence spectrum of (BMVC)₂-8C (tetracation) overlaps with LysoTracker red, LysoTracker blue was used to co-stain with (BMVC)₂-8C (tetracation) and was pseudo-colored red in the fluorescence image. (B) HBC of the BMVC derivatives correlates with lysosomal co-localization. (C) (MVC)₂-8C, which is much larger in molecular size than BMVC but has a similar HBC to BMVC, was similarly retained in the lysosomes of MRC-5 cells. Confocal fluorescence images of CL1-0 (upper panel) and MRC-5 (lower panel) cells incubated with 1 μ M (MVC)₂-8C for 24 h and co-stained with 200 nM LysoTracker red. In all panels, the scale bar is 15 μ m.

Fig. 5. Lipophilicity of 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) derivatives correlates with mitochondrial localization in CL1-0 cells

(A) Confocal fluorescence images of CL1-0 cells incubated with BMVC derivatives with different lipophilicities. “n” corresponds to variable length of alkyl linker. For example, BMVC-4C is the abbreviated name for BMVC derivative with butyl linker to another N-methyl-piperidinium. (B) Confocal fluorescence images of CL1-0 cells incubated with BMVC-8C3O and overlaid with Hoechst 33342 (upper) or MitoTracker red (lower). (C) Fluorescence of the BMVC derivatives with lipophilicities (log P) higher than -2.0 is

observed only in the mitochondria, whereas fluorescence of BMVC derivatives with lipophilicities below -2.0 is detected in the nucleus and in the mitochondria of CL1-0 cells. Structures of all these compounds are in Fig.7

Fig. 6. Subcellular localization differences of 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) between drug-sensitive and drug-resistant cancer cells

(A) Epi-fluorescence images of drug-sensitive MCF-7 (left panel) and drug-resistant MCF-7/ADR (right panel) cancer cells incubated with 5 μ M BMVC. (B) Confocal fluorescence images of drug-sensitive MCF-7 (upper panel) and drug-resistant MCF-7/ADR (lower panel) cancer cells incubated with 5 μ M BMVC and co-stained with 200 nM LysoTracker red. (C) Flow cytometric analysis of the relative fluorescence changes of doxorubicin and BMVC upon 50 μ M verapamil treatment, which inhibited P-glycoprotein, or 0.5 mM L-leucyl-L-leucine methyl ester (LeuLeuOMe) treatment, which increased the lysosomal membrane permeability, of MCF-7/ADR cancer cells. (D) Confocal microscopic images of BMVC in MCF-7/ADR cells upon LeuLeuOMe treatment. Cells were stained with 1% propidium iodide (PI) to distinguish living cells from dead cells.

carbazole

Fig. 7. Structures of BMVC derivatives