2018

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Edwin J. Wiest

Heather Jensen Smith

Michael A. Hollingsworth

Published in final edited form as:

Biochem Biophys Res Commun. 2018 July 02; 501(4): 858–862. doi:10.1016/j.bbrc.2018.05.034.

Met receptor inhibitor SU11274 localizes in the endoplasmic reticulum

Edwin J. Wiest[a,b], **Heather Jensen Smith**[b], **Michael A. Hollingsworth**[a,b,*]

[a]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA

[b]Eppley Institute, Fred and Pamela Buffet Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA

Abstract

We discovered that SU11274, a class I c-Met inhibitor, fluoresces when excited by 488 nm laser light and showed rapid specific accumulation in distinct subcellular compartments. Given that SU11274 reduces cancer cell viability, we exploited these newly identified spectral properties to determine SU11274 intracellular distribution and accumulation in human pancreatic cancer cells. The aim of the studies reported here was to identify organelle(s) to which SU11274 is trafficked. We conclude that SU11274 rapidly and predominantly accumulates in the endoplasmic reticulum.

Keywords

SU11274; Endoplasmic reticulum; Sulfonylurea receptor; Unfolded protein response

1. Introduction

In the course of examining activities of Met, a receptor tyrosine kinase that transduces signals from the extracellular space to the cell interior when its ligand, hepatocyte growth factor (HGF) binds to its extracellular domain [1], we noted that the small molecule inhibitor SU11274, a pyrrole indolinone compound first described in 2003 [2], showed unexpected properties of fluorescence and cellular uptake into specific organelles. SU11274 is reported to inhibit the Met receptor activation with an IC50 of 10 nM as determined in a cell-free assay [3], due to the its ability to bind tightly to the ATP pocket of the Met receptor [2]; however, rapid accumulation of this molecule in specific subcellular compartments has not been previously reported.

*Corresponding author. Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA. mahollin@unmc.edu (M.A. Hollingsworth).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.05.034.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.05.034.

2. Materials and methods

2.1. Cell culture and treatment conditions

S2.013.MUC1F pancreatic cancer cells, derived from a liver metastasis, were cultured in DMEM containing 5% FBS at 37 °C and 5% CO_2 [4]. Cells were grown to 80% confluency prior to experimentation. For live cell experiments, SU11274 (Selleckchem) was applied to S2.013.MUC1F cells at 0.1, 1, and 10 μM (see below). For fixed cell experiments, 2 μM SU11274 was applied for 30 min before cells were fixed for organelle-specific labeling, as described below.

2.2. Organelle labeling in fixed and live cells

Cells were fixed (4% PFA in PBS) and subsequently permeabilized (0.15% Triton-X 100,1% BSA in PBS) prior to primary antibody staining (see below) for 2 h at 4 °C. Cells were subsequently stained with primary antibodies to detect specific intracellular organelles: anti-calreticulin Alexa Fluor 647 (endoplasmic reticulum, 1:100 v/v, Abcam), anti-Lamp1 (lysosomes,1:1000 μg/μL, Abcam), anti-EEA1 Alexa Fluor 647 (early endosomes, 1:50 v/v, Abcam), and anti-giantin (Golgi apparatus, 1:300 v/v, Abcam). The antibody diluent was 1% BSA in PBS with 0.01% azide. For all non-directly conjugated primary antibodies, anti-rabbit Alexa Fluor 647 was applied at 1:3000 (mg/mL) for 1 h at room temperature. All samples were mounted with Duolink Mounting Medium containing DAPI (Sigma-Aldrich) and stored at 4 °C until imaged.

ER-Tracker™ Red (10 nM, ThermoFisher) was used to define the endoplasmic reticulum (ER) and to establish the time course of uptake and accumulation of SU11274 in live cells.

2.3. Confocal laser scanning microscopy

A Zeiss laser scanning microscope (LSM) 800 with Airyscan (Thornwood, NY) and a 40x/1.3 N.A. oil immersion objective lens was used to generate cell images. All imaging parameters (described below) were universally applied across experimental treatment groups. Briefly, images were acquired using sequential scanning of individual fluorophores with a final image size of 1024 × 1024 pixels and scaling of 0.156 μm per pixel. Images collected using bidirectional scanning were additionally averaged 2 to 4 times to optimize signal to noise ratios.

SU11274 was excited using a 488 nm laser line and the resultant emission was collected using a 495–570 nm filter. ER-Tracker™ Red was excited using a 561 nm laser line and the subsequent emission was collected with a 574–633 nm filter. Alexa Fluor 647, used to counterstain primary antibodies targeted to discrete organelles, was excited with a 640 nm laser line with the resultant emission collected using a 646–700 nm filter. DAPI and Hoechst dyes (for detection of fixed and living cell nuclei, respectively) were excited using 405 nm light with emission collected using a 411–488 nm filter.

Spectral profiling of SU11274 (at 32 μM) was performed using a Zeiss LSM 710 (Thornwood, NY). SU11274 was excited using 488 nm light and subsequent SU11274-associated fluorescence intensities were acquired in sequential 10 nm bins collected from

495 to 725 nm. Upon verification of SU11274's spectral properties, SU11274-specific emission was collected using a 495–570 nm filter set.

Given that SU11274 emission could contaminate the ER-Tracker™ Red signal (Fig. 1A), cells were sequentially imaged 3 min after the application of both compounds. We also observed that 488 nm excitation (used to excite SU11274) exhibits a 5% excitation efficiency for ER-Tracker™ Red and restricted ER-Tracker™ Red emission collection such that it was independent of the SU11274 fluorescence detection filter (Fig. 1A). As a final verification of independent imaging of SU11274 and ER-Tracker™ Red, we imaged SU11274 treated cells without ER-Tracker™ Red labeling and determined SU11274 contamination of the ER-Tracker™ Red channel to be 0.1% (±0.06) and therefore unlikely to generate image analysis artifacts. SU11274 is spectrally distinct from the excitation and emission of Alexa Fluor 647.

2.4. SU11274 cellular uptake assay

S2-013. MUC1F cells cultured on glass-bottom microwell dishes (MatTek) were treated with 100 nM ER-Tracker™ Red for 30 min and then rinsed before applying 2 μM SU11274. The cells were then imaged for 15 min at 20 s time intervals. Cells were maintained at 37 °C and 5% CO_2 during live cell imaging. Average, cell-specific SU11274 fluorescence intensities, were normalized to the highest fluorescence intensity observed and plotted as a function of time. The rate of uptake was calculated by dividing the change in fluorescence intensity from consecutive intensity measurements by the increment of time elapsed between measurements.

SU11274-associated mean fluorescence intensities in individual cells and cell-free areas were additionally analyzed to determine the relationship between SU11274 mean fluorescence intensities and SU11274 concentration. SU11274 cell medium concentration was plotted as a function of SU11274 mean fluorescence intensity measured in the cell-free areas.

2.5. Image analysis

For fixed cell analyses, different subcellular organelles were delineated as discrete regions of interest (ROIs). Raw image files were imported into NIH FIJI/Image J for subcellular organelle localization and SU11274 measurements. Briefly, signal-to-noise ratios for Alexa Fluor 647-labeled subcellular structures were optimized using the Methods thresholding algorithm in NIH Image J. Discrete ROIs representing individual whole cells, subcellular organelles, and representative cell-free regions (background control) were assigned in each analyzed image. ROIs ascribed using antibody-labeled cells were applied to SU11274 images to acquire cell-specific measurements of average SU11274 fluorescence intensities of whole cells and within discrete subcellular organelles of each cell.

2.6. Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). Differences between groups were evaluated by a one-way ANOVA followed by student's T-test as warranted.

Differences were considered significant at $p < 0.05$. Error bars indicate standard error of the mean.

3. Results

3.1. Intrinsic fluorescence of SU11274 can be used to measure cell-specific inhibitor uptake

Spectral profiling of the c-Met inhibitor SU11274 identified novel fluorescence excitation and emission properties well-suited for conventional microscopy (Fig. 1A). Although the emission profile of SU11274 is relatively broad (500–700 nm), peak emission occurs at 515 nm and closely resembles that of commercially available green fluorophores such as FITC, CY2, and Alexa Fluor 488. SU11274 may also be used in conjunction with red fluorophores such as ER-Tracker™ Red (Fig. 1A) or Alexa Fluor 647 to characterize inhibitor uptake and accumulation in specific subcellular organelles. SU11274 is a pyrrole indolinone compound containing a sulfonamide group which may facilitate its fluorescent properties, as well as its ability to accumulate within cells (Fig. 1B).

In whole cell SU11274 uptake experiments, SU11274 rapidly accumulated in pancreatic cancer cells (Fig. 1C and D and Video 1). SU11274 uptake was characterized by an early, rapid phase lasting approximately 2 min followed by a longer lasting, slower phase of uptake that leveled off about 10 min after SU11274 application.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.05.034.

3.2. SU11274 differentially accumulates in subcellular organelles

Whole cell SU11274 uptake studies revealed regions of SU11274 accumulation within S2.013. MUC1F cells. SU11274 localization studies were undertaken using antibodies to label specific organelles including the ER, early endosome, the Golgi apparatus, and lysosome identified SU11274 in multiple organelles (Fig. 2). Average SU11274 fluorescence intensities were greatest in the Golgi and lysosomes. However, localization of SU11274 to the ER comprised a significant portion of the total cell volume (41.93 ± 1.8%), followed by the early endosome (17.1 ± 1.4%), Golgi (14.6 ± 1.3%), and lysosome (6.1 ± 0.7%). When average fluorescence intensities were adjusted to compensate for total contribution to whole cellular content, SU11274 accumulation was greatest in the ER (Fig. 2D).

3.3. SU11274 rapidly accumulates in the ER

Because ER-targeting is a potentially very useful drug property, we determined the concentration of SU11274 in both the whole cell and the ER. We quantified the relationship between SU11274 mean fluorescence intensity and SU11274 concentration by plotting the mean fluorescence intensities measured in ROIs representing the cell-medium as a function of SU11274 concentration applied to the cells (Fig. 3A). We further defined the relationship between SU11274 intensity and concentration ([SU11274]) by the equation [SU11274] = 8.0749 × SU11274 mean fluorescence intensity - 1.365 using arbitrary fluorescence intensity units (A.U.). Estimates of the concentration of SU11274 in whole cells and the ER revealed

greater accumulation in the ER, relative to the whole cell, at all concentrations of SU11274 (Fig. 3B).

4. Discussion

The ER is involved in biosynthesis of lipids, folding and assembly of proteins, and regulation of calcium signaling [5]. Loss of function of the ER is related to several diseases, including cystic fibrosis, diabetes mellitus, Alzheimer's, and Parkinson's [5]. SU11274 accumulates rapidly in cells (Fig. 1 and Video 1), specifically in the ER region (Fig. 2). In fact, after treating cells with 100 nM SU11274 for only 3 min, we found that the cellular SU11274 concentration was nearly 100 times that of the cell medium, and the concentration in the ER region was over 350 times that of the cell medium (Fig. 3).

SU11274 shares a structural feature, a phenyl sulfonamide group, with glibenclamide (the ER-targeting moiety of ER-Tracker™ Red) and glimepiride (Supplementary Fig. 1). These drugs are known to bind sulfonylurea receptors of ATP sensitive-potassium channels [6,7]. It may be that SU11274 interacts with the ER by way of this channel. Discovered in 1942, sulfonylureas are the most common drug used in the treatment of diabetes mellitus [8]. Glibenclamide and glimepiride, second generation sulfonylureas, act on β-pancreatic cells by binding and inhibiting the sulfonylurea receptor of ATP sensitive-potassium channel which stops the inflow of potassium ions into the cells. This causes depolarization of the cell membrane and results in an influx of calcium ions which leads to a release of insulin [8]. Interestingly, SU11274 has been shown to directly impact β-cells. SU11274 caused β-cell death and loss of β-cell mass in rats after two weeks of treatment [9]. However, this stands in contrast to the impact of glimepiride on β-cells. In a six-month clinical study, glimepiride was found to enhance β-cell secretory capacity after six months of treatment [10].

Though sulfonylureas and SU11274 affect cells differently, a chemical moiety they share may be a useful tool to target drugs specifically to the ER. Cancer and cystic fibrosis patients would possibly benefit from ER-targeted therapy. In most types of cancer, the tumor microenvironment is limited in oxygen and glucose, so oxidative ER protein folding and glycosylation-assisted ER protein folding are impaired. This leads to activation of the unfolded protein response (UPR). Cancer cells often rely on UPR for growth and survival [5]. Targeting the ER in B cell malignancies may be especially advantageous. B-chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM) cells have more developed ER networks than normal lymphocytes [11]. In addition, these cells rely on the unfolded protein response (UPR) for plasma cell differentiation [11].

Some proteins crucial to UPR activity, such as X-box binding protein 1 (XBP1), glucose-regulator protein 78 kDa (GRP78), and inositol-requiring enzyme 1 (IRE1) have been targeted for the treatment of breast cancer, myeloma, melanoma, and atherosclerosis [12–14]. It may be that modifying such a drug with a phenyl sulfonamide group would cause it to accumulate in the ER region, resulting in greater efficacy. Such targeted therapy would allow decreased dosage and decreased toxicity. Further examination of the contribution of elements from the chemical structure of SU11274 should provide insight into chemical moieties that enable future ER-targeting drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by a grant from the National Cancer Institute's SPORE in Pancreatic Cancer P50CA127297 and Edwin Wiest was recipient of fellowship awards from the National Cancer Institute (NCI T32009476) and the UNMC Structural Biology and Molecular Biophysics Training Program Graduate Assistance in Areas of National Need (GAAN) fellowship funded by the U.S. Department of Education, (P200A12023). We thank Janice A. Taylor and James R. Talaska of the Advanced Microscopy Core Facility at the University of Nebraska Medical Center for providing assistance with confocal microscopy and the Nebraska Research Initiative and the Eppley Cancer Center for their support of the Core Facility. Support for the UNMC Advanced Microscopy Core Facility was provided by the Nebraska Research Initiative, the Fred and Pamela Buffett Cancer Center Support Grant (P30CA036727), and an Institutional Development Award (IDeA) from the NIGMS of the NIH (P30GM106397). The NIH SIG funded instrument LSM 710 Zeiss Confocal Microscope (NIH S10RR027301) was used in this work.

The experiments were performed and analyzed by Edwin Wiest. Michael Hollingsworth provided guidance in experimental design, data analysis and manuscript writing. Heather Jensen Smith assisted in data analysis and manuscript editing.

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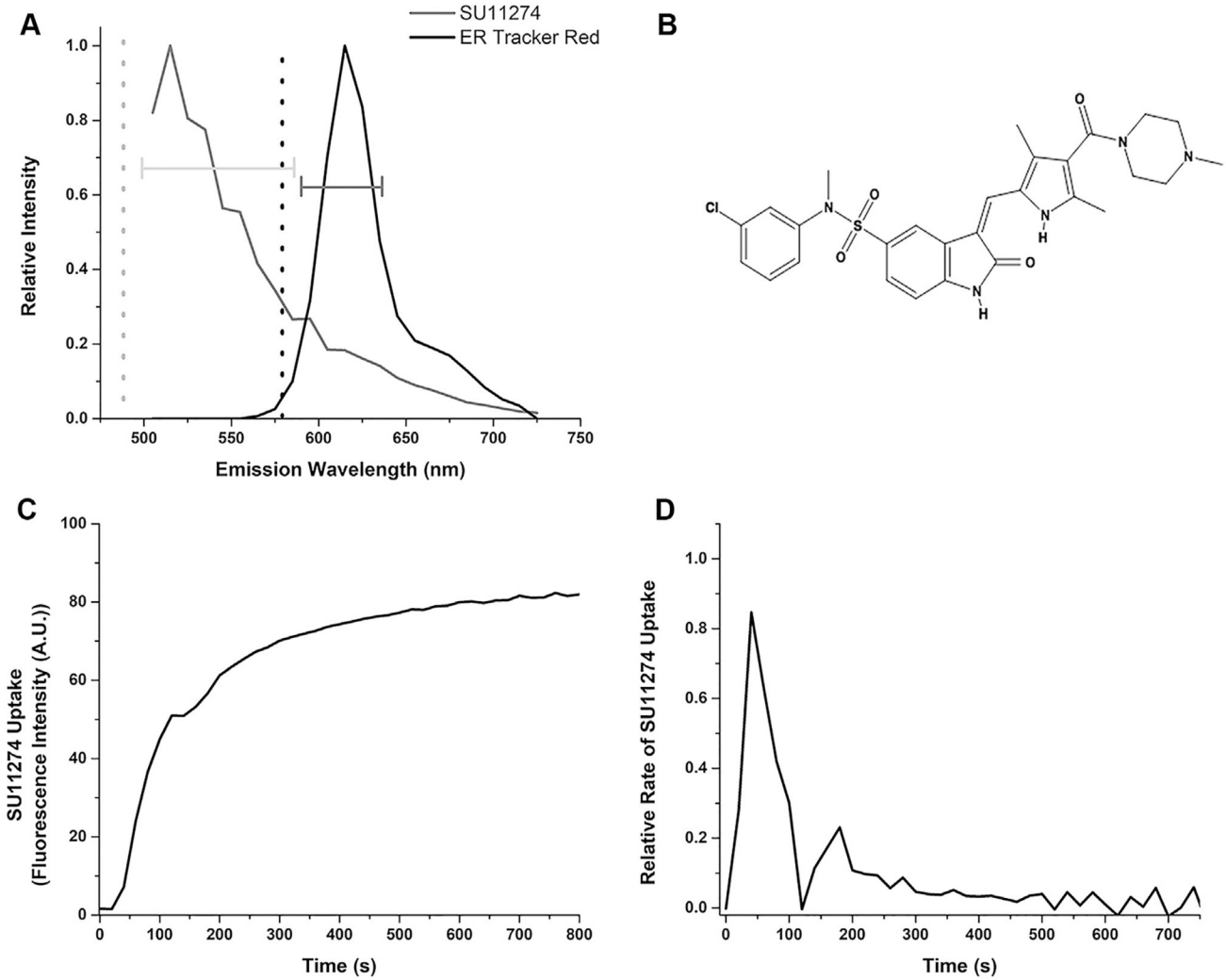

Fig. 1. The intrinsically fluorescent c-Met inhibitor, SU11274, rapidly accumulates in cells.

(A) SU11274 excitation (488 nm, grey dotted line) and emission profile can be spectrally separated from ER-Tracker™ Red excitation (561 nm, black dotted line) and emission using SU11274- and ER-Tracker Red™-filter sets (grey, black bars, respectively). (B) Structure of SU11274 ($C_{28}H_{30}ClN_5O_4S$). (C) Cell-specific SU11274 uptake, measured by fluorescence intensity, is rapid in S2-013.MUC1F cells. (D) Relative rates of SU11274 uptake by S2-013.MUC1F are indicative of a biphasic uptake.

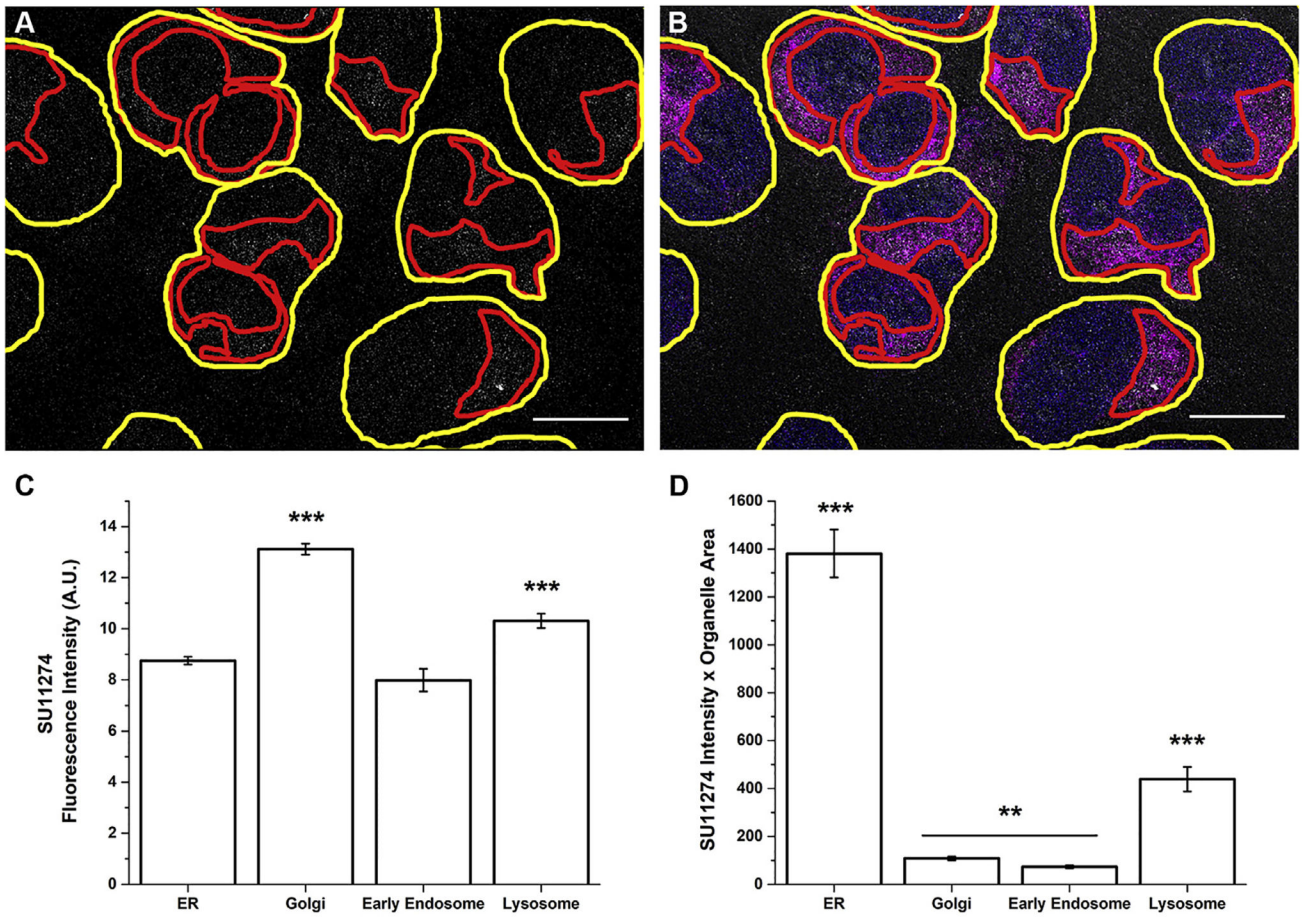

Fig. 2. SU11274 differentially accumulates in subcellular organelles.

SU11274-associated fluorescence (white) in whole cells (outlined in yellow) (A) and in the calreticulin (purple)-labeled ER (outlined in red) (B). Nuclei are labeled with DAPI (blue). (C) SU11274-associated mean fluorescence intensities in ROIs representing antibody-labeled organelles. (D) Each SU11274-associated mean fluorescence intensity measurement was multiplied by the area of the ROI in which the measurement was made. Scale bars represent 20 μm $^{**}p < 0.01$, $^{***}p < 0.001$.

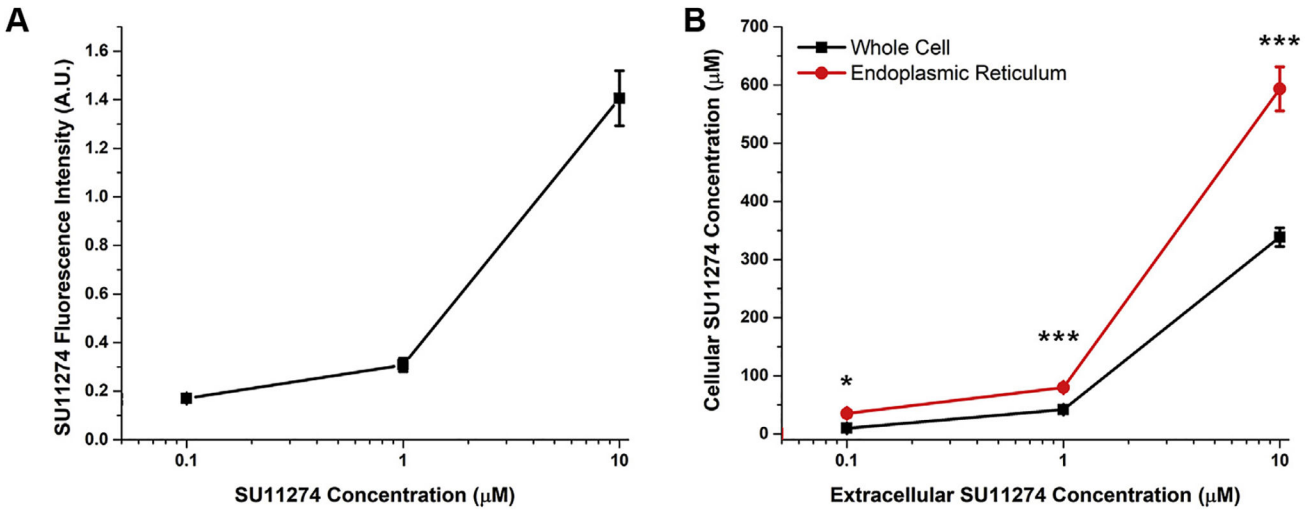

Fig. 3. SU11274 rapidly accumulates in the ER.

(A) SU11274 fluorescence intensity is positively correlated with SU11274 concentration.

(B) SU11274 actively accumulates in cells and the ER. SU11274 accumulation is greatest in the ER at all extracellular SU11274 concentrations. *p < 0.05, ***p < 0.001.