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Drug accumulation into single drug-sensitive and drug-resistant prostate cancer cells conducted on the single cell bioanalyzer

单细胞生物分析仪分析单一药敏和耐药前列腺癌细胞的药物蓄积作用

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ABSTRACT

Multidrug resistance (MDR) occurs in prostate cancer, and this happens when the cancer cells resist chemotherapeutic drugs by pumping them out of the cells. MDR inhibitors such as cyclosporine A (CsA) can stop the pumping and enhance the drugs accumulated in the cells. The cellular drug accumulation is monitored using a microfluidic chip mounted on a single cell bioanalyzer. This equipment has been developed to measure accumulation of drugs such as doxorubicin (DOX) and fluorescently labeled paclitaxel (PTX) in single prostate cancer cells. The inhibition of drug efflux on the same prostate cell was examined in drug-sensitive and drug-resistant cells. Accumulation of these drug molecules was not found in the MDR cells, PC-3 RX-DT2R cells. Enhanced drug accumulation was observed only after treating the MDR cell in the presence of 5 μM of CsA as the MDR inhibitor. We envision this monitoring of the accumulation of fluorescent molecules (drug or fluorescent molecules), if conducted on single patient cancer cells, can provide information for clinical monitoring of patients undergoing chemotherapy in the future.

摘要

多药耐药经常发生在前列腺癌症患者中，表现为癌细胞泵出化疗药物产生耐药性。多药耐药抑制剂例如环孢霉素A(CsA)能阻止化疗药的泵出并增加其在细胞中的蓄积。使用单细胞生物分析仪的微流体生物芯片可以监测该蓄积。该仪器已经应用于测量药物在前列腺单细胞中的蓄积如柔红霉素（DOX)和荧光标记的紫杉醇（PTX）。此篇文章考察了药物在同一前列腺癌药敏和耐药细胞流出的抑制作用。但是，在多药耐药细胞(PC−3RX−DT2R)细胞中没有发现这些药物。仅仅在5uM多药耐药抑制剂CsA存在的情况下才观察到药物的蓄积作用。我们预见，监测荧光分子（药物或荧光分子）在细胞中的蓄积作用，如果应用在

患者的癌细胞分析上，在未来将会为正在化疗的患者提供极为重要的临床监测信息。

INTRODUCTION

Resistance to a broad range of anticancer drugs, termed as multidrug resistance (MDR), is most often associated with the drug-pumping action of drug transporter proteins on cancer cell membranes, leading to little drug accumulated in cells [1]. The poor response to chemotherapy in patients with prostate cancer (PC) led us to study drug accumulation in cultured human prostate cancer cells within a microfluidic biochip. MDR inhibitors can stop the drug transporters from pumping, resulting in the enhancement of accumulated drug in the cells [2]. In this work, the amount of accumulated drugs enhanced by MDR inhibitors was monitored on a single-cell bioanalyzer using the same-single-cell analysis (*SASCA*). This method has proven to work on human leukemia cells [3, 4], conducted on a full microscopic imaging and photometric measurement system [5]. In this paper, we report how the microfluidic method can be used to measure prostate cancer cells on a newly developed compact SCB equipment.

简介

对大多数抗癌药耐药，或称多药耐药（MDR），其作用机制一般认为与癌细胞膜药物转运蛋白将药物泵出有关，导致了细胞内几乎没有药物蓄积。化疗药物在前列腺癌患者治愈率较低，因此我们使用微流体生物芯片研究培养的人体前列腺癌细胞的蓄积作用。多药耐药抑制剂能阻止药物转运蛋白泵出药物，增强了细胞中的药物蓄积作用。在该项实验中，使用同一单细胞分析法用单细胞生物分析仪定量监测了由MDR抑制剂引起的药物蓄积。该法具有完整的显微图像和光学测量系统，已经证明可用于人体白血病细胞分析。在此文中，我们报道了使用最新发明的微型单细胞生物分析仪测定前列腺癌细胞的微流体方法。

THEORY

In order to overcome the issue of cellular variations, *SASCA* in the drug accumulation mode (*SASCA-A*) was performed in which the same cancer cell was used as both the control and test cell.

原理部分

为了克服细胞差异引起的问题，我们使用了SASCA药物蓄积模式（SASCA−A）对同一癌症细胞同时作为对照品和测试品进行了测试。

In the *SASCA-A* approach, drug accumulation was measured in the trapped cell first treated with anticancer drug or fluorescent molecule in the absence of an MDR inhibitor (control experiment), followed by drug accumulation measured on the same single cell treated with drug or fluorescent molecules in the presence of an MDR inhibitor (the test). The microfluidic *SASCA-A* method can provide time-dependent drug transport data as well as cell morphological information. Besides, only a small amount of cells and reagents are needed to confirm the findings.

在SASCA−A模式中，首先在缺少MDR抑制剂的情况下，测量捕捉到的使用抗癌药或荧光分子处理过的细胞中的药物蓄积（对照试验），随后测量在MDR存在情况下，同一细胞使用药物或荧光分子后的药物蓄积(测试实验)。微流体SASCA−A方法可以提供实时性的药物转运数据，同时，提供细胞的形态信息。此外，仅需要小量细胞和试剂来确认结果。

EXPERIMENTAL

实验部分

Cell Culture. The drug resistant cell line PC3-RX-DT2R has been previously developed [6]. This cell line and their parent cell line PC-3 were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 UmL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin and 2 mM glutamine. For subculture, the cells were detached using trypsin-EDTA and re-seeded.

细胞培养。使用以前研发的细胞系PC−3RX−DT2R[6]。该细胞系和其母细胞系PC−3用RPMI−1640介质培养，该介质包括10%小牛血清（FBS), 100UmL^{-1}青霉素，100μgmL^{-1}链霉素，2mM谷胱氨酸。传代培养后，细胞使用胰蛋白酶-EDTA脱离并重新接种。

Reagents. Doxorubicin (DOX) and cyclosporine A (CsA) were obtained from Sigma-Aldrich. Oregon green 488-labeled paclitaxel (PTX), RPMI-1460 medium, trypsin/EDTA (0.025%), penicillin/streptomycin (PEN/STR) and fetal bovine serum (FBS) were obtained from Life Technologies.

试剂。柔红霉素（DOX)和环保菌素(CsA)来自Sigma-Aldrich；俄勒冈绿标记的紫杉醇（PTX），RPMI-1460介质, 胰蛋白酶/EDTA (0.025%), 青霉素/链霉素(PEN/STR)和小牛血清 (FBS) 来自 Life Technologies。

Single Cell Bioanalyzer (*SCB*). In this work, the single cell bioanalyzer (*SCB*) was developed (see Fig. 1) to measure drug accumulation on cancer cells in response to the application of MDR inhibitors to block MDR transporters' pumping activity. The cells were introduced into a single-cell biochip mounted on an open-frame translation stage (Semprex Corp., Campbell, California). A single cell was selected and trapped within the U-shaped retention structure. The accumulated drug in a single cell was excited by a blue laser (Frankfurt Laser Co., Germany) with the intensity partially reduced using a neutral density filter (NDF). The total fluorescence and background were monitored by translating the chip, and hence the attached

cell, back and forth, as previously described [7]. Here, the translating procedure was automated using a robotic arm (SOC Robotics, North Vancouver). Optical imaging was conducted using a portable TV colormonitor (Digital Prism).

单细胞生物分析仪（SCB).
在该项试验中，我们使用了单细胞生物分析仪（SCB）（见图1）测量癌症细胞的药物蓄积，用来反映使用MDR抑制剂阻断MDR转运蛋白的泵活性。细胞被引入放置在敞开式平移台的单细胞生物芯片中（SemprexCorp., Campbell,California)。单细胞被选择捕捉到U型保留结构中。单细胞中蓄积的药物被蓝色激光激发(FrankfurtLaser Co.,Germany)，使用中等强度的滤片（NDF），部分地减少了其强度。如前所述，前后移动芯片，因此细胞测定样品荧光强度和背景[7]。移动过程由机械手自动完成(SOCRobotics,NorthVancouver)。光学图像由可携带式彩色电视显示器记录(Digital Prism)。

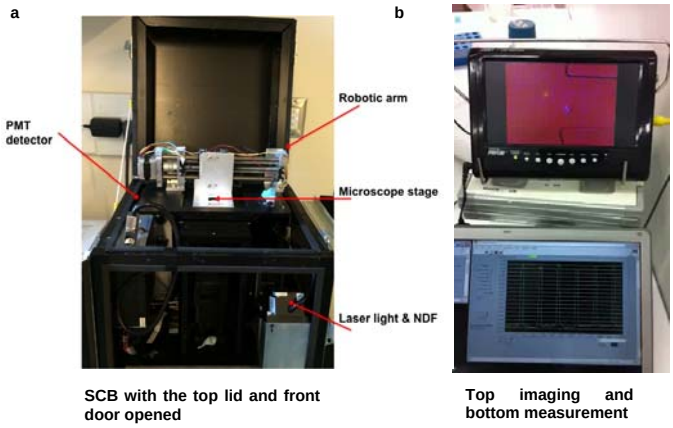

Fig. 1. Single cell bioanalyzer (SCB) shown in (a) and the associated TV monitor for optical imaging of the prostate cancer cell and the computer screen for fluorescent measurement of 6 peaks above background shown in (b).
图一.单细胞生物分析仪（SCB)(a)附带的记录前列腺癌细胞光学图像的电视监视器和电脑荧光屏显示六个峰荧光信号和背景信号测量（b).

The fluorescent emission in yellow was detected by a photomultipler (PMT) module (Hamamatsu, Japan), with the output voltage collected by a computer interface card (National Instruments). The data was displayed and stored using the Labview software [8]. The *SCB* has several advantages for single cell analysis assays: First, the SCB is suitable for use when only a small number of cancer cells, such as patient cells, is available. Second, the laser light source can be conducted with a small spot size to measure the cell; this alleviates the need of an aperture to define the location of the cell and the surrounding while the chip is being translated automatically. Third, this instrument is designed to be compact so that it can be used in research and clinical settings, and its inner surface is black-anodized (Surf-Tech Industries) and the outer walls are powder-coated (Altech Custom Coaters).

黄色发射荧光被光电倍增管(PMT)检测(Hamamatsu,Japan)，输出电压由电脑接机卡收集(NationalInstruments) Labview软件显示和储存数据[8]。单细胞生物分析仪对单细胞分析有几项优势：首先，该仪器适用于小量癌细胞分析，如患者细胞。第二，激光源的使用可测量极小范围的细胞，避免了在芯片自动转移的过程中使用狭缝来定性细胞和周围的位置。第三，该仪器内部黑色阳极涂层(Surf−TechIndustries)，外部粉末涂层(AltechCustomCoaters)，设计如此紧凑，特别适用于研究和临床。

PDMS Microchip. The microfluidic biochip layout was designed using L-edit (Tanners) and the photomask was printed on a plastic film (Coles Lithoprep). The chip was made of a 15 mm × 15 mm polydimethylsiloxane (PDMS) slab using a previously described process [9]. The PDMS slab was sealed to a 0.17-mm thick glass cover slip. Fig 2a depicts such a chip with the channels and chamber filled with a blue dye [10]. As shown in Fig. 2b, the chip is composed of three channels, three reservoirs and one chamber containing the cell retention structure. As shown in Fig. 2b, the left and right reservoirs (1 and 3) serve as the inlet and waste reservoirs, respectively; whereas, the middle reservoir 2 is used for drug delivery. The cells were introduced from the left side reservoir, and the cells flowed from the left channel to the right via the central cell chamber. By adjusting the liquid levels of these reservoirs, a single cancer cell was led into the cell retention structure located in the middle of the central chamber, as reported previously [11].

PDMS微流体芯片。微流体生物芯片被设计使用L-edit (Tanners)，光掩模被打印在塑料膜上(ColesLithoprep)。该芯片是由一个15mm×15mm的聚二甲基硅氧烷（PDMS）使用先前描述的过程制备[9]。PDMS片与0.17mm厚的玻璃片密封。图2a显示了这样的芯片，通道和细胞池充满了蓝色染料[10]。如图2b显示，芯片是由3个通道，3个储液池和一个具有细胞保留结构的细胞池组成。如图2b所示，左和右储液池（1和3）分别作为进样和废液池；然而，中间储液池2作为药物传输。正如以前报道，细胞从左边池引入，进入左边通道，通过中间室进入右边池。通过改变这些池的液体容量，单一癌细胞被引入中间室的细胞保留结构内[11]。

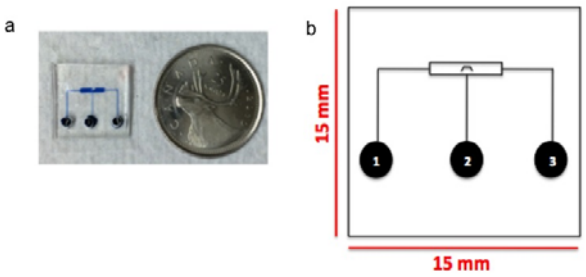

Fig. 2. The microfluidic chip. (a) Image of the microchip (1.5 cm × 1.5 cm) filled with a blue food dye. A Canadian quarter (25 cents) was placed next to the chip for size comparison. (b) The schematic showing reservoir 2 used for drug delivery; whereas reservoirs 1 and 3 served as the cell inlet and waste, respectively.

图二.微流体芯片。（a)充满蓝色食物染料的微流体芯片图像（(1.5cm×1.5cm)。加拿大25分硬币置于芯片旁作为比较。（b)示意图显示储液池2用于药物递送;而储液池1和3分别担任细胞入口和废弃物。

Single Cell Retention and On-Chip Drug Accumulation Study. Fig. 3 shows the retention of a prostate cancer cell in the single-cell chip. Simultaneous optical observation and fluorescent measurement were conducted. The significance of single-cell retention is to examine drug accumulation on one and the same single cell. It is because the same cell is used as the test cell as well as the control cell since different cancer cells have different MDR pumping properties. This method is called same single cell analysis in the drug accumulation mode (*SASCA-A*), as previously reported [3, 4]. In this regard, the trapped single cell was treated with the fluorescent molecule (DOX or PTX) in the absence of CsA (as a control), and in the presence of this MDR inhibitor (as the test). If the cell were multidrug resistant (MDR), the initial drug accumulation was low, but adding MDR inhibitors could increase the accumulation of the chemotherapeutic drug. But if the cell was not MDR, there was high drug accumulation, without the need of adding any inhibitors.

单细胞保留和芯片药物蓄积研究

图3表明了前列腺癌细胞在单细胞芯片的保留。光学观察和荧光测定被同时进行。单细胞保留的重要意义在于测定药物在一个并且同一个细胞的药物蓄积。因为不同的癌细胞具有不同的MDR泵药特性，这样，同样一个细胞既用于测试，并又作为对照细胞，具有重要的意义。如以前报道，该方法被称为药物蓄积模式的相同单细胞分析法(*SASCA−A*)[3,4]。因此，捕捉到的单细胞分别在缺少CsA的情况下（作为对照品）和在MDR抑制剂存在的情况下（测试品）通过荧光分子(DOXorPTX)的处理。如果细胞是多药耐药的（MDR），起始剂量很低，添加MDR抑制剂后可以增加化疗药的蓄积。但是如果细胞不是MDR的，不用添加任何抑制剂，就具有较高的药物蓄积。

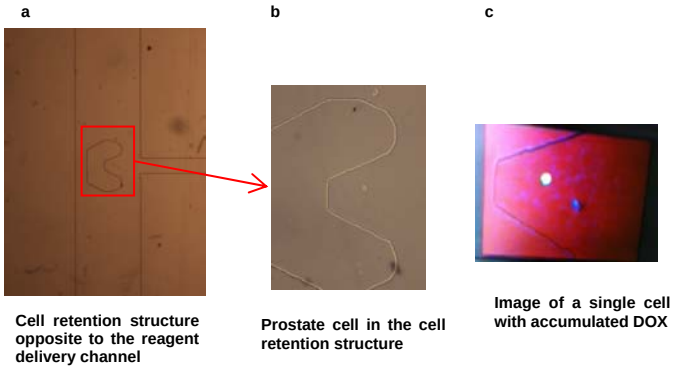

Fig. 3. Cell retention in a microfluidic biochip. (a) The cell retention structure is located in the chip to retain cells. (b) Closeup of a prostate cancer cell retained in the cell retention structure. (c) Optical imaging (under red light) of a single PC-3 RX-DT2R cell with fluorescent emission in yellow due to DOX accumulation.

图三.微流体生物芯片细胞保留。（a)芯片上用来保留细胞的细胞保留结构。（b)细胞保留结构中的前列腺癌细胞的特写图（c)光学图像（红光下），单一PC−3RX−DT2R细胞由于DOX蓄积引起的黄色荧光。

After being trapped inside the cell retention structure, the cell was settled for 15 min. to allow for cell attachment. Then, fluorescence measurement was started when the fluorescent drug molecules were introduced. Doxorubicin (DOX) was the first drug used since it was a multidrug resistance (MDR) substrate mediated by the P-gp protein on the cell membrane [1]. The permeability-glycoprotein (P-gp), encoded by the MDR-1 gene, is perhaps the most well characterized drug efflux pump in MDR cancer cells [12]. DOX also has an inherent fluorescence (λ_{ex}= 470 nm; λ_{em}= 585 nm). However, for paclitaxel, fluorescent labeling by Oregon Green; λ_{ex}= 492 nm; λ_{em}= 524 nm) was required. During fluorescent measurement, the chip was translated to the right and left by the robotic arm in order to obtain signals from the cell and the background in a consecutive manner. Subtraction of the background from the cell signal gave the corrected signal representing the drug concentration inside the cell.

细胞被引入保留结构中，停留15分以便细胞附着。然后，引入荧光药物分子，开始荧光测量。柔红霉素(DOX)被首先使用，因为该药是细胞膜P−gp蛋白调节的多药耐药(MDR)替代物[1]。渗透性−糖蛋白(P−gp)，由MDR−1基因编码，可能是最好定性的MDR癌症细胞的流出泵。DOX可自身发荧光(λ_{ex}=470nm;λ_{em}=585nm)。可是，紫杉醇需要俄勒冈绿标记（λ_{ex}=492nm;λ_{em}=524nm)。在荧光测量过程中，芯片由机械臂左右移动，连续获取细胞和背景信号。细胞信号除去背景信号所得到的矫正信号即为细胞内的药物浓度。

RESULTS AND DISCUSSION

After a single MDR prostate cancer cell (PC-3 RX-DT2R) was retained inside the biochip, the *SASCA-A* measurement was conducted, followed by background correction, as shown in Fig. 4. In the first step, the MDR cell was treated with 70 μM of DOX for 800 s. As shown in the inset, only a weak cellular fluorescence signal was obtained when DOX accumulation occurred in the absence of a MDR inhibitor. In the next step, the same cell was treated with DOX (70 μM) in the presence of 10 μM of CsA at 800 s. The cellular fluorescence intensity started to rise and reached 0.1 V at 1700s (see inset), and the cell became yellow (see Fig. 3c). The intensity continued to rise and reached 0.3 V when CsA (10 μM) was included as the P-gp inhibitor to enhance drug accumulation.

The effectiveness of accumulated drug enhancement by CsA is indicated by the fold increase, which is defined as the ratio between the fluorescence signal of the inhibitor-blocked cell and that of the unblocked cell. Adding 10 μM of CsA caused the single cell fluorescence to increase by 7.5 fold.

结果和讨论

当单一MDR前列腺癌细胞(PC−3RX−DT2R)保留在生物芯片后，开始*SASCA-A*测量，随后进行背景矫正，见图4.

首先，MDR细胞用70μMDOX处理800秒。如内图所示，DOX蓄积发生在MDR抑制剂缺失的情况下仅获得微弱的细胞荧光信号。接下来，同一细胞在10μM的CsA存在的情况下用DOX(70μM)处理800秒。细胞荧光强度开始升高在1700秒时达到0.1V，细胞变为黄色（见图3c)。当CsA (10μM)的P−gp抑制剂引入后作为增强药物蓄积，荧光强度继续升高达到0.3V。由CsA增强的药物蓄积作用是成倍增长的，被定义为抑制剂阻断和未阻断的细胞荧光信号之比。加入10μM的 CsA引起单细胞荧光信号增加7.5倍。

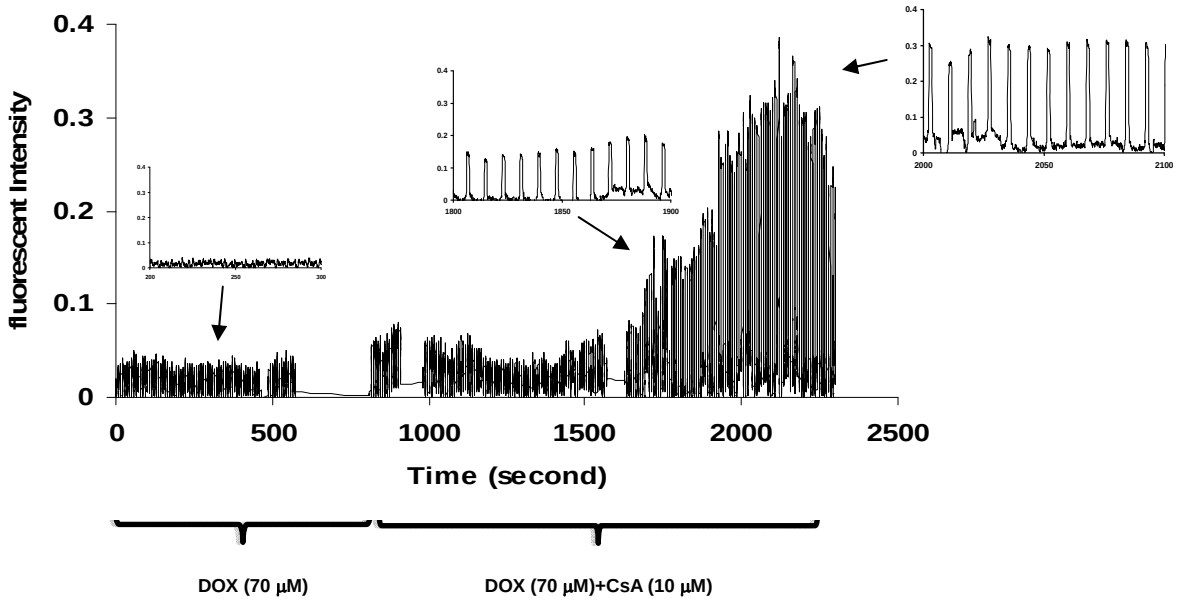

Fig. 4. Accumulation of DOX into a single PC-3 RX-DT2R cell. The initial cellular fluorescence intensity was low (0.04 V). With anti-cancer drug (70 μM of DOX) added at 800 s, the intensity started to rise at 1700 s to 0.1 V and the cell became yellow. Then, the fluorescence intensity continued to rise and reached 0.3 V.

图四.单细胞PC−3RX−DT2R中DOX的蓄积。开始时细胞荧光强度较低(0.04V)。随着在800s时加入抗癌药(70μM DOX)，荧光强度开始增加，在1700s时上升到0.1V，细胞变为黄色。然后，荧光强度继续上升到0.3V.

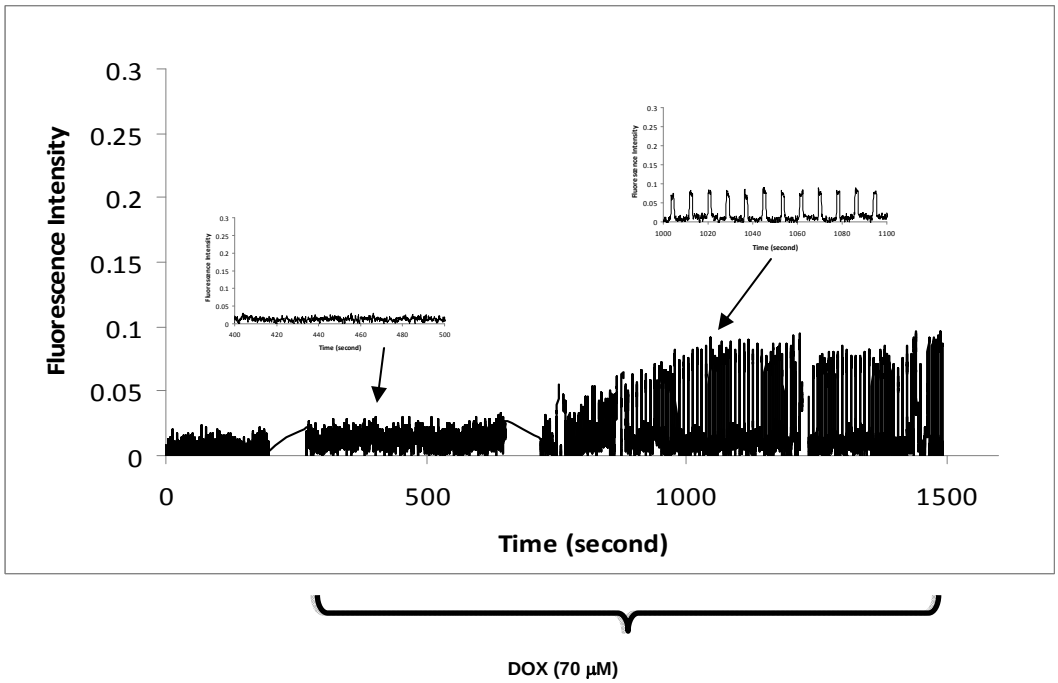

Fig. 5. Drug accumulation in a single PC-3 cell, with added DOX (70 **μM***) at 300 s. The cellular fluorescence intensity started to rise to 0.02 V at 700 s, and continued to rise and reached 0.1 V.*
图五. 单一PC-3 细胞在300 s加入DOX (70 **μM***)的药物蓄积,. 荧光强度在700s上升到0.02V,并继续升至0.1V.*

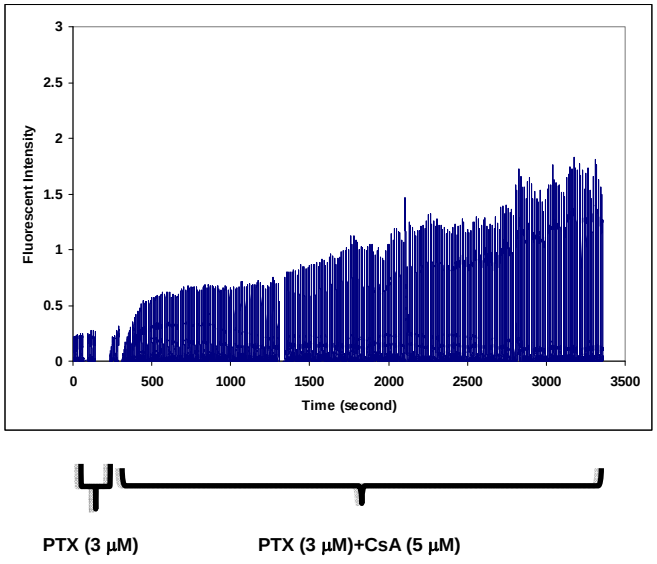

Fig. 6. Accumulation of **PTX** *(3* **μM***) into a single PC-3 cell, with CsA (5* **μM***) mixed with PTX at 235 s. The cellular fluorescence was 0.2 V without adding CsA, but the intensity rose to 1.5V after CsA was added. The cell started to glow brightly green at 1300 s when the fluorescent intensity reached 0.5 V.*

图六. 单一PC-3 细胞中PTX (3 μM)的蓄积，235s 时 CsA (5μM)与PTX混合。细胞荧光强度为0.2V,加入CsA后升至1.5V.在1300s时细胞开始发出亮绿光，同时，荧光强度达到0.5V.

Similar *SASCA-A* experiments were performed on the parent cells (PC-3). The cell was treated with 70 μM of DOX, and the drug accumulation became high even without the addition of CsA, see Fig. 5.

PC-3 cells were also used for the accumulation experiment of paclitaxel labeled by OG-488 (PTX). Figure 6 shows the measurement of accumulation of PTX into a single PC-3 cell. When the cell was treated with 3 μM PTX, only a small amount of cellular fluorescence was measured. Thereafter, the cell was treated with 3 μM PTX mixed with the P-gp inhibitor (5 μM CsA), and the drug accumulation became much higher, and the cell glowed fluorescently in green. The single-cell experiment was repeated several times with similar results. Another PC-3 cell that was treated with PTX alone for as long as 1.7 h did not produce cellular fluorescence greater than 0.2 V. So we concluded that for PTX accumulation into even the PC-3 cell, CsA was required for inhibition of drug pumping mediated by P-gp. This observation is consistent with the finding that P-gp is expressed at relatively high levels in PC-3 prostate cancer cells [13].

对患者细胞(PC-3)做了*类似的SASCA−A*实验。细胞用70μM DOX处理，药物蓄积变得很高，甚至在没有添加CsA的情况下，见图5。
PC−3细胞也被用作OG−488(PTX)标记的紫杉醇蓄积实验。图6显示了单一PC−3细胞中PTX的蓄积曲线。当细胞用3μMPTX处理后，仅小量细胞荧光被监测到。因此，细胞用3μMPTX与P−gp抑制剂(5μMCsA)同时处理，药物蓄积变得较高，细胞发出绿色荧光。该单细胞实验重复多次得到类似结果。另一个PC−3细胞单独被处理长达1.7h,没有产生超过0.2V的细胞荧光。因此，我们得出结论，甚至PC−3细胞内的PTX蓄积，需要CsA抑制由P−gp调节的药物泵。这一观察与发现，与P−gp在前列腺癌PC−3细胞中的高水平表达一致[13]。

CONCLUSION

The single cell bioanalyzer (SCB) has allowed us to measure drug accumulation in single prostate cancer cells. DOX was accumulated in the PC-3 cell, but not in the PC-3 RX-DT2R (the MDR subline). Only when CsA was mixed with DOX that there was substantial drug accumulation in the PC-3 RX-DT2R cell. On the other hand, for PTX accumulation into even a PC-3 cell, CsA was required. These results will be useful for planning for the measurements of drug accumulation in rare cells in the future. These cells, such as circulating tumor cells (CTCs) isolated from the blood samples of castrated-resistant prostate cancer patients, can be of high clinical significance. To achieve this, the patient blood samples, after removal of red blood cells, can be introduced into the disposable biochip. *SASCA-A* measurement is then performed on the SCB and the results are obtained in an hour. This result will be compared with the CTC counts obtained from the CellSearch system.

结论

单细胞生物分析仪(SCB)使我们能够测量单一前列腺癌细胞的药物蓄积。DOX在PC−3细胞中蓄积,却不在PC−3RX−DT2R中蓄积(MDR细胞系。仅仅当CsA与DOX混合后，大量的药物蓄积才在PC−3RX−DT2R细胞中观察到。另一方面，PC−3细胞中的PTX蓄积需要CsA的参与。这些结果对未来罕见细胞的药物蓄积是很有用的。这些细胞，如从阉割性前列腺癌患者血样分离出的循环肿瘤细胞(CTCs)具有重要的临床意义。为此，患者血样在分离了红细胞后，被引入一次性生物芯片中，随后使用单细胞生物分析仪开始*SASCA−A*测量，一小时之内获得结果。结果将与CellSearch获得的CTC计数结果对比。

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Nomenclature

ABC: ATP-binding cassette; ATP: adenosine triphosphate; CsA: Cyclosporine A; DOX: Doxorubicin; MDR: Multidrug resistance; NDF: Neutral density filter; PDMS: Polydimethylsiloxane; P-gp: Permeability-glycoprotein; PMT: Photomultiplier; PTX: Oregon green 488-labeled paclitaxel; SASCA-A: Same-single-cell analysis in the accumulation mode; SCB: Single Cell Bioanalyzer;