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Application of LC/MS/MS in the quantitation of SU101 and SU0020 uptake by 3T3/PDGFr cells

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Abstract

SU101 or leflunomide, has been studied extensively because of its anti-cancer and immunomodulating properties. The parent isoxazole compound is converted in vitro and metabolized in vivo to an open ring isomeric form, SU0020. Several pharmacological activities have been reported for the parent and metabolite compounds including inhibition of platelet-derived growth factor (PDGF)-mediated signaling for the parent compound and inhibition of de novo pyrimidine biosynthesis for the metabolite. The inhibition of PDGF-mediated signaling and the anti-tumor properties have been ascribed to the parent compound. In spite of its short plasma half-life of the parent molecule, SU101 can be administered intermittently in animal tumor models and retain efficacy. Therefore, the relationship between plasma levels of SU101 and its efficacy in tumor-implanted immuno-compromised mice is not well established. This study was conducted to assess the concentration of SU101 in 3T3/PDGFr α and β cells (NIH3T3 mouse fibroblasts engineered to overexpress human PDGFr α or β) to better understand the cellular levels of SU101 and SU0020. Two strains of 3T3/PDGFr cells (α and β) were incubated with 1, 25, and 100 μM concentrations of SU101 for 1, 6, 24, and 48 hours. Quantitation of SU101 and SU0020 in these cell lines was achieved by a specific and sensitive liquid chromatography-tandem mass spectrometry (LC/MS/MS) method. Interestingly, in both α and β cell lysates SU101 was much more concentrated than SU0020. The greater concentration of SU101 versus SU0020 that was observed may be due to the preferential partitioning of SU101 into the cells and this shows that significant levels of the parent drug can reach the pharmacological site of action for inhibition of PDGF receptors. The data suggest that the conversion of SU101 to SU0020 is much slower in these cells than in the incubation media. © 2002 Published by Elsevier Science B.V.

Keywords: SU101; Leflunomide; Liquid chromatography-tandem mass spectrometry; Cell stability; Chemical conversion; Platelet-derived growth factor

1. Introduction

SU101 or Leflunomide ($C_{12}H_9N_2O_2F_3$, 270.2 Da), has been studied extensively as an immunomodulating agent [1–5] and more recently

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as an anticancer drug [6,7]. This compound has shown potent inhibition of tumor growth in numerous models of xenografts including those of glioma, ovarian, and prostate origin, as well as metastatic models [6]. The parent isoxazole compound is converted in vitro and metabolized in vivo to an open ring isomeric form, SU0020 (Fig. 1). SU101 demonstrates anti-tumor effects through inhibition of signaling mediated through PDGF receptor phosphorylation, inhibition of DNA synthesis and cell cycle progression which typically follow the interaction of PDGF with its receptor [6]. It is important to note that the PDGF receptors are transmembrane proteins with an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. Therefore inhibition of the enzymatic function of the PDGF receptors by small molecules requires that they cross the cell membrane in sufficient concentration. SU0020 has been reported to inhibit de novo pyrimidine synthesis [8–10] via activity on dehydro-orotate dehydrogenase.

SU101 is rapidly converted to SU0020, as reported by earlier investigations [7,11,12]. This rapid biotransformation to SU0020 is most notable in plasma. For example, at the end of a 6-h infusion of SU101 at 24 and 72 mg/kg in rats, plasma concentrations of SU0020 were 100-fold higher than that of SU101 [12]. The fact that SU101 rapidly disappears from systemic circulation raises the question of what entity is responsible for the anti-tumor efficacy. The observation that SU101 is readily detectable in tissues such as brain and implanted tumor xenografts, even though SU101 is more than two orders of magnitude lower than SU0020 in plasma immediately post administration, suggests that the parent compound may indeed demonstrate efficacy [13,14].

Recently, a novel LC/MS/MS method was developed which measures both SU101 and SU0020 in rat plasma. This method effectively preserves the integrity of SU101 during sample processing and utilizes the high sensitivity of mass spectrometric detection (LOQ = 0.5 ng/ml). SU101 pharmacokinetic parameters were thus measured accurately for the first time in the rat [12].

With a modified LC/MS/MS method, we conducted a cell stability study of SU101 to further investigate the mechanism of action of SU101 at the cellular level. NIH3T3 mouse fibroblasts, engineered to overexpress human PDGF receptor α or β (3T3/PDGFr α or 3T3/PDGFr β), were incubated with 1, 25 or 100 μM SU101 for 1, 6, 24 or 48 h. The levels of both SU101 and SU0020 in the cells and in the 1 μM incubation media were measured by this LC/MS/MS method. A HPLC–UV method was used to measure SU101 and SU0020 in the 25 and 100 μM incubation media in order to avoid the excessive dilution that would be necessary if the LC/MS/MS method was used.

2. Experimental

2.1. Chemicals

Certified ACS grade acetonitrile, ammonium citrate dibasic, ammonium acetate, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). HCl was certified grade from Fisher Scientific. Distilled water was purified on a Milli-Q water purification unit (Millipore, Bedford, MA).

Citrate buffer was prepared by combining 7 ml 0.1 N HCl with 3 ml 0.1 M ammonium citrate

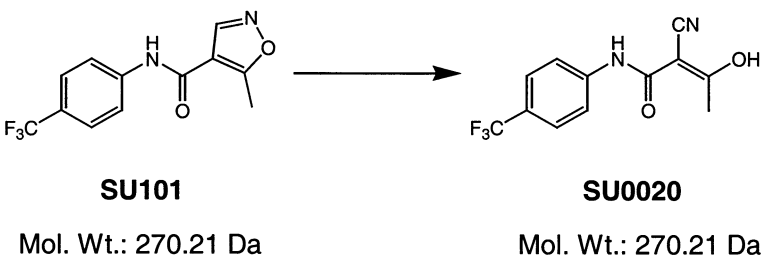

Fig. 1. Biotransformation of SU101 to SU0020.

dibasic. The pH of this buffer was about 1.5. The HPLC mobile phase A consisted of 10% acetonitrile, 90% water and 10 mM ammonium acetate. Mobile phase B consisted of 90% acetonitrile, 10% water and 10 mM ammonium acetate. The same mobile phases were used for both the HPLC assay and the LC/MS/MS assay.

2.2. SU101 cell incubation and sampling

Following a 24-h incubation in growth medium (Dulbecco's modified Eagle's medium + 10% calf serum), 125,000 3T3/PDGFr cells (α or β) were incubated with 1, 25 and 100 μM SU101 in starvation medium (Dulbecco's modified Eagle's medium + 0.1% BSA) in 12-well plates at 37 °C. The incubation was stopped after 1, 6, 24 or 48 h by putting the plates on ice. A 500-μl aliquot of the medium was transferred to a microcentrifuge tube and stored at −20 °C pending analysis. The remaining medium was discarded. The cells were washed twice with ice-cold phosphate buffered saline. Then, 500 μl acetonitrile/citrate buffer (90:10) was added to lyse the cells. The mixture was incubated on ice for 5 min and the plates were shaken for 30 s. The cell lysate was transferred to a microcentrifuge tube on dry ice. The samples were stored at −20 °C.

2.3. Sample preparation

For HPLC analysis, the standards were prepared by diluting the standard solutions of SU101 and SU0020 (1:1 mixture) at 2, 5, 10, 20 or 50 μg/ml in acetonitrile/citrate buffer (90:10) with an equal volume of stock starvation medium. Quality controls (QCs) at 4, 7 and 40 μg/ml were prepared in a similar manner. SU101 incubation media samples were diluted with equal volume of acetonitrile/citrate buffer (90:10). The standards, QCs, and the incubation media samples were vortexed, centrifuged and the supernatants were transferred and stored at −20 °C pending analysis. The samples were warmed to room temperature and vortexed immediately prior to analysis.

For LC/MS/MS analysis, SU101 and SU0020 standards (1:1 mixture) were prepared by diluting 1 μg/ml stock solution in acetonitrile with acetonitrile/citrate buffer (90:10) to achieve concentrations of 0.5, 1, 2, 5, 10, 20 and 50 ng/ml. QCs at 1.5 and 40 ng/ml were prepared in a similar manner. Most cell lysates were directly analyzed without any dilution. For some 100 μM cell lysates, dilution with acetonitrile/citrate buffer (90:10) was necessary to bring the concentrations within the standard curve range. Both the standards and cell lysates were stored at −20 °C. Immediately prior to analysis, the samples were warmed up to room temperature. After vortexing and centrifugation, ~100 μl of the sample was transferred to a HPLC vial for analysis.

2.4. High performance liquid chromatography assay

The HPLC–UV assay was developed to measure SU101 and SU0020 concentrations in the 25 and 100 μM SU101 incubation media (≥ 2 μg/ml). This assay was performed on a Hewlett Packard HP1100 series HPLC system with detection at 254 nm. The column was 50×4.6 mm^2 YMC ODS-AQ C-18 with 5 μm particles. The gradient started at 30% mobile phase B and reached 40% B in 2 min. Then, it was immediately changed to 60% B. After 3 min the gradient reached 100% B and was held there for 1.5 min. It was then decreased to 30% B and equilibrated at 30% B for 1.5 min before the next analysis. The flow rate was 1 ml/min and the injection volume was 50 μl. This method was not sensitive enough to measure SU101 and SU0020 concentrations at < 2 μg/ml.

2.5. Liquid chromatography-tandem mass spectrometry assay

The LC/MS/MS assay was used to determine the concentrations of SU101 and SU0020 in cell lysates and incubation media below 2 μg/ml. The LC/MS/MS assay was performed on a PE Sciex API 365 triple quadrupole mass spectrometer with a turbo ion spray source. The mass spectrometer was operated in the negative ion MRM mode, detecting both SU101 and SU0020 at 269/82 mass channel. Prior to MS/MS detection, samples were subjected to LC separation on a Shimadzu LC-10AD system equipped with a Perkin Elmer Series 200 autosampler. A Keystone BDS Hypersil C18

column (150 × 3 mm^2, 5 μm particles) was used to achieve the separation. In the first 3 min, the LC gradient was held at 5% mobile phase B. Then it was quickly changed to 60% B and dwelled there for 3 min. It took another 3 min to reach 100% B and was maintained at 100% B for 3 min. The LC resumed its initial gradient and equilibrated for 2 min before the next run. For the first 5 min in each run, the elution was diverted to waste. The flow rate was set at 350 μl/min and 20 μl was injected.

3. Results and discussion

3.1. Extraction of SU101 and SU0020 from cells

To assess the extraction efficiency of SU101 and SU0020 from cells, three different solvent systems were examined: methanol, acetonitrile/citrate buffer (9:1), and mobile phase B. A pilot study was conducted where 1 or 100 μM SU101 was incubated with 3T3/PDGFr β cells for 1 h. The cell lysates were extracted with the three aforementioned solvents. Methanol, which is conventionally used for lysing cells, produced ion chromatographic peak intensity comparable to the acetonitrile/citrate buffer. Mobile phase B, which contained 10 mM ammonium acetate, promoted the formation of SU101 and SU0020 negative ions before sample injection, and consequently enhanced the mass spectrometric detection by improving the ionization efficiency. However, since the acetonitrile/citrate buffer system can effectively prevent the conversion of SU101 to SU0020 as determined in an earlier study [12], we selected this system to obtain cell lysates in this study. For the samples containing analyte(s) concentrations at above the high limit of quantitation, dilution was made by using the acetonitrile/citrate buffer solvent system. The extraction efficiency of the acetonitrile/citrate buffer system was examined at 2 and 20 ng/ml. Standard solutions containing equal concentrations of SU101 and SU0020 prepared in the respective solvents were mixed with control cells or incubation medium and subsequently analyzed with LC/MS/MS. The peak areas of both SU101 and SU0020 from the matrices were compared to that of the corresponding neat standard solutions. At 2 ng/ml, the extraction efficiency for SU101 in α cells, β cells, and incubation medium was 92.7, 89.3 and 101.6%, respectively, and for SU0020 was 112.0, 102.3 and 105.6%, respectively. At 20 ng/ml, the extraction efficiency for SU101 in α cells, β cells, and incubation medium was 107.6, 95.0 and 106.2%, respectively, and for SU0020 was 115.1, 109.4 and 121.6%, respectively. Since the extraction efficiencies for both analytes were close to 100%, neat standard solutions were used to construct the calibration curve for the analysis of cell lysates and 1 μM incubation media.

3.2. LC/MS/MS and HPLC method performance

Since both SU101 and SU0020 have the same precursor/product mass channels, the two analytes were separated chromatographically. Fig. 2a was obtained from β cell lysate free from the analytes. There was no interference from the matrix with the SU101 signal. The weak peak at SU0020 retention time was the carry-over from a previous injection. This low level of carry-over did not interfere with the analysis of SU0020 since at the lowest level of standard curve (0.5 ng/ml), SU0020 signal was five times higher (Fig. 2b). Fig. 2c was obtained from β cell lysate incubated with 100 μM SU101 for 24 h, showing the ion chromatogram of SU0020 (RT = 7.3 min) and SU101 (RT = 11.0 min). The two compounds were well separated.

For LC/MS/MS, linear curves were observed in the range of 0.5–50 ng/ml. The average ($n = 9$) correlation coefficients were 0.9982 and 0.9984 with coefficients of variation (CV) of 0.134 and 0.127% for SU101 and SU0020, respectively. The limit of quantitation was 0.5 ng/ml. At this level, the signal-to-noise ratio for both analytes was found to be higher than 5:1. For SU101 measurement at all levels of standards and QCs, the accuracy was within 10% ($n = 9$) from the target concentration. The coefficient of variation (CV) was $\leq 12\%$ ($n = 9$). For SU0020 standards and QCs, the accuracy was also within 10% ($n = 9$) from the target concentrations and the CV was $\leq 11\%$ ($n = 9$).

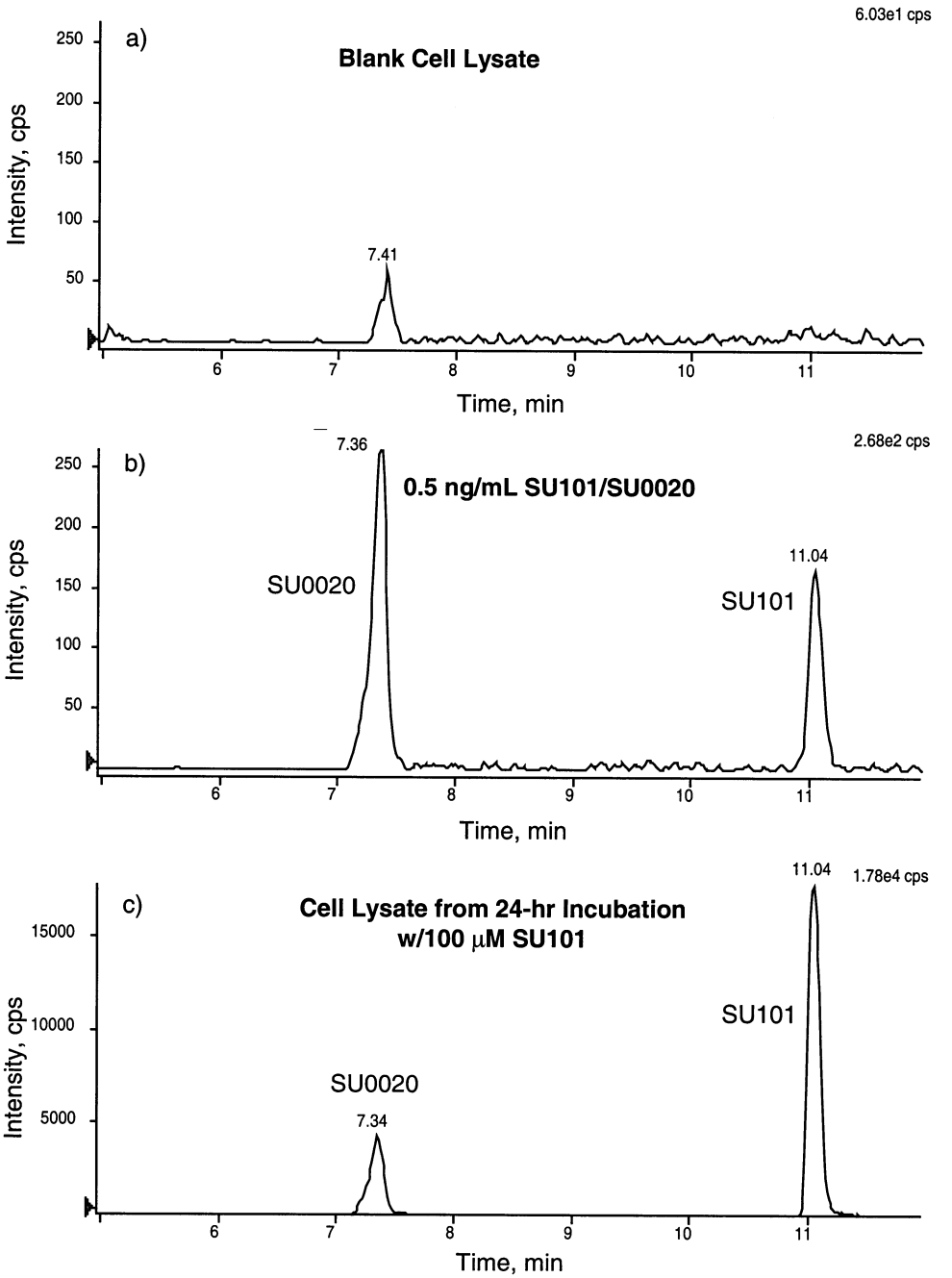

Fig. 2. LC/MS/MS ion chromatograms of SU101 and SU0020 obtained from: (a) blank β cell lysate; (b) 0.5 ng/ml SU101 and SU0020 standard; and (c) β cell lysate after 48-h incubation with 100 μM SU101.

The standard curve for the HPLC method was in the range of 2–50 μg/ml with a limit of quantitation 2 μg/ml. The average ($n = 8$) correlation coefficients were 0.9998 and 0.9998 with coefficients of variation (CV) of 0.0226% and 0.0173% for SU101 and SU0020, respectively. Showing in

Fig. 3 are the chromatograms of SU101 (RT = 4.76 min) and SU0020 (RT = 2.02 min) in blank incubation medium, at 2 μg/ml and 100 μM after 6 h of incubation. This method was selective and there were no interfering peaks from the media. All the SU101 standards and QCs were within 9%

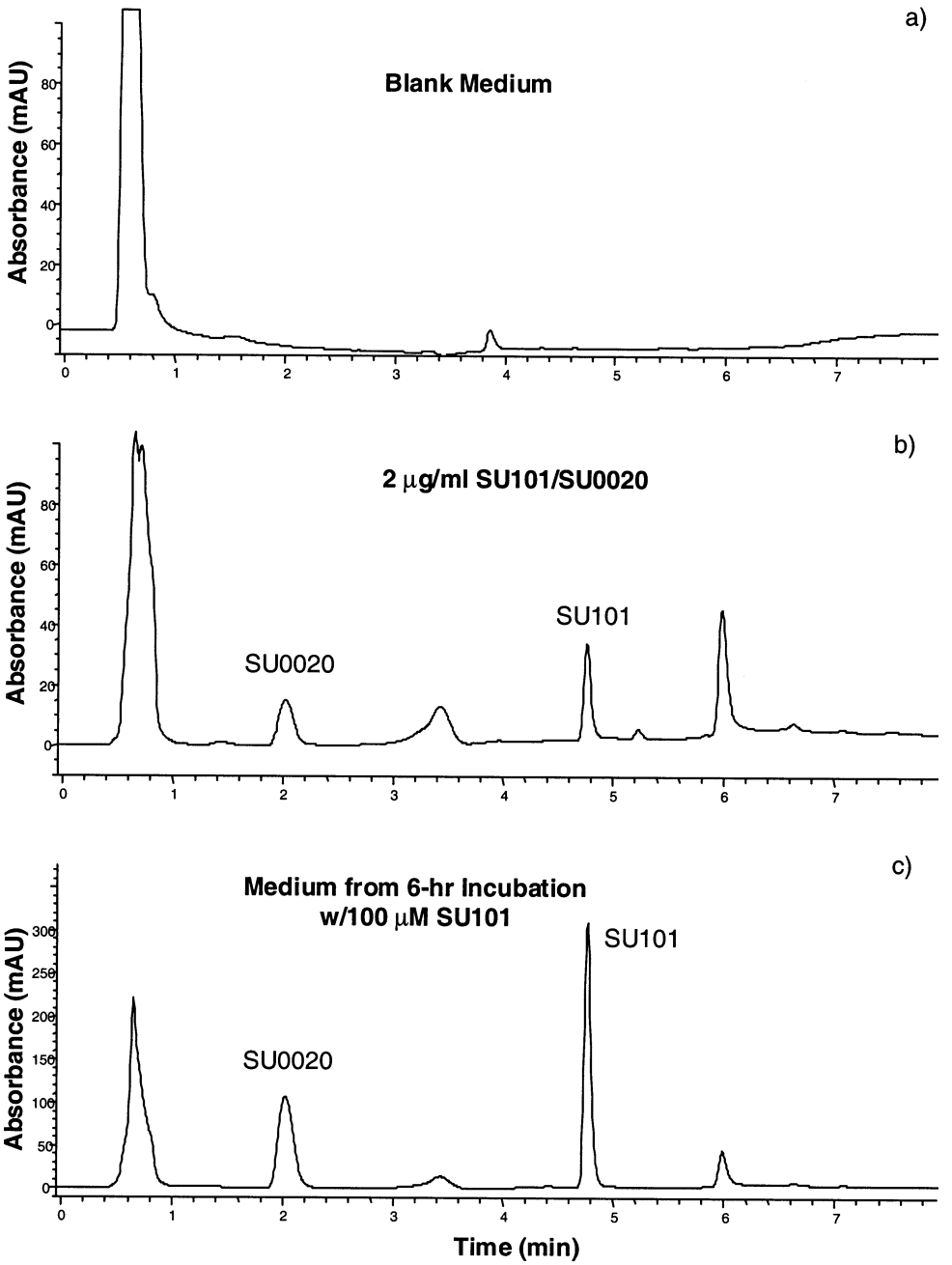

Fig. 3. HPLC–UV chromatograms of SU101 and SU0020 obtained from: (a) blank starvation medium; (b) 2 μg/ml SU101 and SU0020 standard; and (c) 100 μM β cell medium after 6-h incubation.

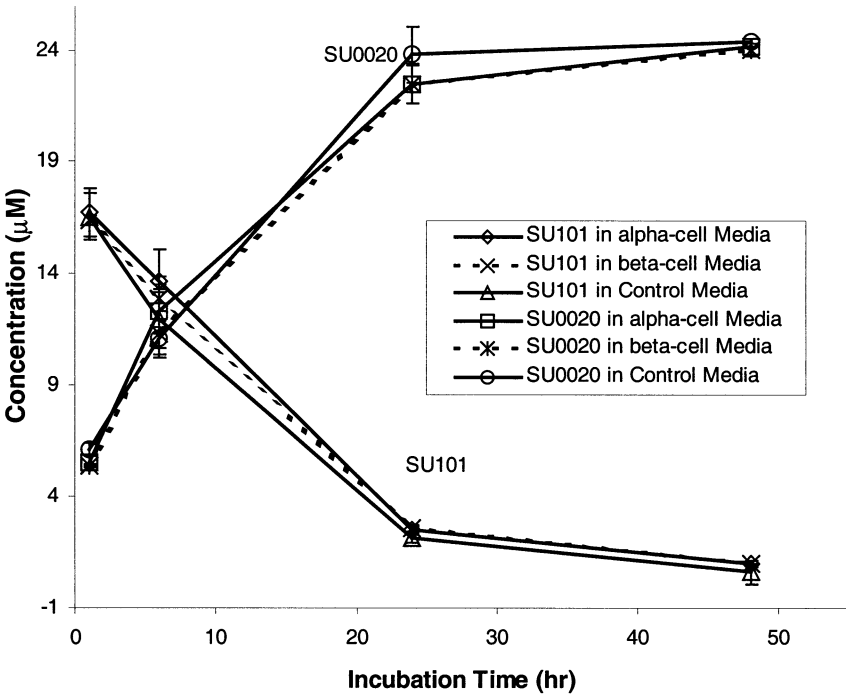

Fig. 4. SU101 and SU0020 medium concentrations following incubation of 3T3/PDGFr cells with 25 μM SU101 for the times indicated. The media were analyzed by the HPLC method.

of the corresponding nominal concentrations. The CV was $\leq 10\%$. All the SU0020 standards and QCs were within 11% of the target concentrations and the CV was $\leq 11\%$.

3.3. SU101 and SU0020 levels in cells and media

SU101 and SU0020 concentrations in the incubation media are illustrated in Fig. 4 as represented by incubating 3T3/PDGFr α and β cells with 25 μM SU101. Each data point is the average of three measurements from three individual incubations. In the incubation media, SU101 concentration decreased rapidly. SU0020 concentration, on the other hand, increased rapidly over the course of the incubation. At 24 h, $\sim 90\%$ of SU101 were converted to SU0020. At 48 h only 4% of SU101 remained in the incubation media. This rapid conversion of SU101 to SU0020 in the incubation media (pH ~ 7.4) was independent of cell type, since media from both α and β cells as well as the control media (where no cells were present during the entire course of incubation) showed the same results. Similar patterns were observed in the media of 1 and 100 μM SU101 incubations.

The corresponding cellular levels of SU101 and SU0020 are shown in Fig. 5. The cellular concentrations of SU101 and SU0020 were calculated based on the assumption that the mean 3T3 cell volume is about 1.5 pl [15] and the total number of cells was estimated to be 1.25×10^5 in each incubation well. The total cell volume, therefore, was:

$$1.5 \times 10^{-12}\ (\text{l}) \times 1.25 \times 10^5 = 1.875 \times 10^{-7}\ (\text{l})$$

Since the cells were lysed with 0.5 ml of solvent, the quantity of an analyte in each measurement was $C \times 0.5$ (ng), where C (ng/ml) was obtained from the LC/MS/MS measurement. Thus, the corresponding cellular level of that analyte was:

$$C \times 0.5 \times 10^{-3}/(270 \times 1.875 \times 10^{-7})$$
$$= 9.877 \times C \text{ with the unit of } \mu\text{M}$$

where 270 is the molecular weight of SU101 and SU0020. For example, at 6-h incubation with 25 μM SU101, the level of SU101 in β cell lysates was measured as 30.4 ng/ml. Consequently the derived cellular level was $9.877 \times 30.4 = 300$ μM. In cell lysates, the relative abundance of SU101 and SU0020 exhibited a pattern very different from that of the incubation media. As demonstrated in Fig. 5, SU101 cellular levels peaked at 6-h incubation and then gradually decreased as time progressed. SU0020 cellular levels increased only gradually as incubation time increased. In general, in both α and β cell lysates, SU101 was much more concentrated than SU0020. Similar trend was observed following incubations with 1 and 100 μM SU101. The highest concentrations of SU101 detected in α (β) cells were 13.7 (17.3), 236 (300), and 614 (590) μM after 6 hours of incubation with 1, 25, and 100 μM concentrations of SU101, respectively. The highest cell concentration for SU0020 was found to be 177 μM in α cells after 48 h of incubation with 100 μM of SU101. At 24 h, SU0020 cellular concentration was less than 25% of that of SU101 for cells incubated with 25 and 100 μM SU101, even when the incubation media were dominated by SU0020. The difference between SU101 levels in α and β cell lysates was statistically insignificant.

It is interesting to note that both SU101 and SU0020 were detected in the phosphate buffered saline solution that was used to wash the cells after incubation. In the first wash solution, the concentration ratio of SU101 to SU0020 was 2.9 ± 0.6 ($n = 3$), close to that in the incubation medium (1.4 ± 0.1, $n = 3$). In the second wash solution, this ratio was 9 ± 1 ($n = 3$), close to that in the cell (14 ± 3, $n = 3$). It was possible that the second wash may extract some analytes from the cells, however, it did not appear to preferentially extract one of the analytes.

The greater concentration of SU101 versus SU0020 in the cells that was observed may be due to the preferential partitioning of SU101 into the cells. Once inside the cells, the conversion of SU101 to SU0020 is very slow compared to the conversion rate in the incubation media. The observation that the cellular concentration of SU101 was much higher than that in the media was of great importance. For instance, after incubation with 100 μM SU101 for 48 h, less than 4 μM

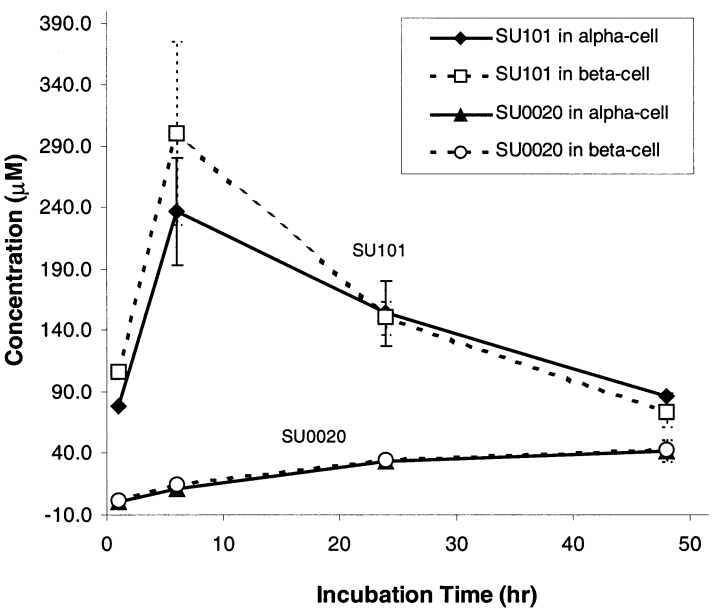

Fig. 5. SU101 and SU0020 cellular concentrations following incubation of 3T3/PDGFr cells with 25 μM SU101 for the times indicated. The cell lysates were analyzed by the LC/MS/MS method.

SU101 remained (and conventionally measured) in the incubation media whereas more than 300 μM of SU101 remained in cells. This shows that in vitro, significant levels of SU101 can reach the pharmacological site of action for inhibition of PDGF receptors.

The results from this study suggest that the anti-tumor efficacy of SU101 in human and animal tumor models [6,7] exerted by the parent compound can be found at the site of action, the catalytic domain of the PDGF receptor inside the cell. This study also demonstrated the high capability of liquid chromatography/tandem mass spectrometry over HPLC–UV in the study of SU101 anti-cancer mechanism of action at the cellular levels.

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