Proteomic Analysis of a Human Prostate Cancer Cell Line after Incubation with a Novel Somatostatin14 Derivative

ZHAOXU LIU1,2, MARCELA MARQUEZ1, STEN NILSSON1, ANDERS R. HOLMBERG1 and AYODELE ALAIYA1

1Karolinska Institute, Urologic Oncology Group, CCK R8/3, 17176 Stockholm, Sweden; 2School of Nursing, Shandong University, Jinan, 250012, China

Abstract. Background: Derivatives of somatostatin (sms) are sometimes used in the treatment of hormone-refractory prostatic cancer, in spite of modest results in controlled clinical studies. The optimal use in this context remains to be determined. The human prostatic cancer cell line LNCaP has been used in several previous proteomic analysis studies, which confirmed that sms indeed can affect the protein expression in this cell line. Proteomic analysis is an important tool to increase the understanding of how sms affects the protein expression of the tumor cell. Materials and Methods: In this in vitro study, a new sms14 derivative, smsdx, a conjugate between sms14 and dextran, was incubated with an LNCaP cell culture. Sms14 was used as the positive control. Proteomic analysis, using rapid mini two-dimensional electrophoresis, was performed to determine the effects on protein expression. Results: Marked quantitative differences were observed in the protein expression profiles in smsdx-treated LNCaP cells compared to negative control cells (untreated cells). Sets of 63 (yet unidentified) protein spots were differentially expressed. The difference was statistically significant (Mann-Whitney analysis). The 63 dataset was used to accurately discriminate control cells from smsdx-treated cells using hierarchical cluster analysis. Both similarities and differences in protein expression were observed between smsdx- and sms14-treated cells. Conclusion: Smsdx is a new sms14 derivative with long in vivo half-life and pan receptor affinity. Sms14-like effects on the protein expression of LNCaP cells seem to be preserved in the construct. The results convey new information about this potentially useful compound. Further studies are now in progress to identify the affected proteins.

Prostate cancer is the most frequently diagnosed male malignancy in the industrialized world. The prognosis of early stage well-differentiated prostate cancer is favourable. Unfortunately, patients often present with extra-prostatic invasion at the time of diagnosis (1). The majority of the patients with metastatic prostate cancer respond favourably to hormonal therapy (androgen ablation or treatment with anti-androgens). However, the therapeutic effect of hormonal therapy is transient and the disease usually becomes hormone-refractory i.e. it no longer responds to hormonal therapy. This condition has poor prognosis. The molecular mechanisms underlying the transition from androgen-sensitive to androgen-resistant prostate cancer are not fully understood. Hormone-refractory prostatic cancer (hrpc) does express somatostatin (sms) receptors (SSTR) and sms analogues (octapeptides, octreotide) have been tested, however with modest results (2-4). Hrpc expresses mainly SSTR subtype1 and, since the octapeptides lack affinity to this subtype (affinity only to SSTR2 and SSTR5), it could be one explanation for the disappointing results to date. Sms derivatives with a high affinity to SSTR1 have, therefore, been considered as potentially useful for the treatment of hrpc.

Recently, we reported about a sms derivative based on natural sms (sms14) with high affinity to all five SSTR subtypes. The construct (smsdx) is a conjugate between sms14 and dextran (5, 6). Several studies (in vitro, animal studies) have shown that sms analogues can inhibit the growth of experimental prostate cancers (7, 8). In vitro studies, on human hrpc cell lines deriving from the LNCaP cell line, suggest that sms exerts a direct inhibitory effect on the cell proliferation and protein secretion through activation of phosphotyrosyl protein phosphatases (9).

Proteomic analysis using classic high-resolution two-dimensional electrophoresis (2-DE) combined with highly sensitive mass spectrometry is capable of simultaneous analysis and characterization of a complex mixture of protein expression patterns. Several studies have described
changes in protein expression between normal, benign prostatic hyperplasia and prostate cancer (10-16).

In order to elucidate and learn more about the cellular effects of smsdx, proteomic analysis was used. The LNCaP cell line was incubated with sms14 and smsdx and the protein expression was subsequently analysed. The results convey new information about this potentially useful compound.

Materials and Methods

Cell culture. The LNCaP human prostate cancer cell line (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM glutamine and 100 IU/ml penicillin-100 µg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO2. The medium was changed twice a week and the cells were trypsinized and subcultivated once a week. Smdx was prepared as described previously (5). Smds14 was from Ferrng, Kiel, Germany.

The cell culture was treated with smsdx or with sms14 for 3 days, 1 nM per day, as described by Brevini (9). The controls were untreated (negative) and cells treated with sms14 (positive).

Sample preparation. The cell line was incubated with smsdx and the preparation of the total cell lysate were performed as previously described by Franzen (17). Protein determination was made using the Pierce BCA protein assay reagent (Rockford, IL, USA).

SDS-PAGE (Bio-Rad) were run using a Criterion Dodeca cell gel apparatus (Bio-Rad). A total of 4 gels were run per sample group.

2-D electrophoresis. The samples were diluted to a total volume of 250 µl, 0.2% Pharmalyte, 8 M urea, 0.3% DTT, 2 M CHAPS and a trace of bromphenol blue (Sigma). An amount of 100 µg of protein was loaded on each strip via rehydration using non-linear pH 3-10 Ready Strip IEF strips (Bio-Rad, Hercules, CA, USA). Focusing was carried out for a total of 45,500 Vh in a PROTEAN IEF cell (Bio-Rad). Precast gels (12.5% homogenous Tris-HCl Criterion) SDS-PAGE (Bio-Rad) were run using a Criterion Dodeca cell gel apparatus (Bio-Rad). A total of 4 gels were run per sample group. The electrode running buffer was 25 mM Tris, 192 mM glycine, 0.1% w/v SDS. Gels were run at 250 V for approximately 1 hour until the bromophenol blue marker had reached the bottom of the gel at a temperature of approximately 15°C. The proteins were visualized by silver staining as described by Rabilloud (18).

Gel scanning and Image analysis. 2-DE gels were scanned at 100 µm resolution (12-bits/pixel) using a GS-710 laser densitometer (Bio-Rad). The data were analysed using PDQuest software Version 7. (Bio-Rad). After auto-detection of all protein spots, gel-images were carefully edited. The individual proteins quantities were expressed as ppm of the total integrated optical density. All 2-DE maps were matched and evaluated independently. Quantitative data sets were generated and subjected to hierarchical cluster analysis using the J-Express software. The clustering pattern is represented diagrammatically as a dendrogram (19). The variables used in the cluster analysis were selected using both the Mann-Whitney signed-ranked test and the Student’s t-test, (p<0.05) for statistical analyses between control and sms-treated cells. The methodological reproducibility of the 2-DE analysis was determined using group correlation analysis (20). Briefly, the total optical density is directly correlated to the total protein concentration.

Minor differences in gel loading, running conditions and silver staining may affect sample comparisons and the 2-D gel reproducibility. Four gels were run from each treatment group and comparisons of the intensity of matched spots between 2-DE gels were performed using the correlation coefficient analysis. A correlation coefficient was measured between 2 gels based on the optical densities of the same spots in the 2 gels being compared. A correlation coefficient of 1 implies that the 2 samples being compared are identical. In a group consisting of 4 samples, a maximum of 6 pair-wise comparisons are possible. The average correlation coefficient among the smsdx samples was 0.85 (n=6 gel pairs, range 0.80 – 0.92).

Results

A high resolution and reproducibility of protein spots from all samples were obtained with the criterion mini 2-DE gels. The correlation coefficients were between 0.85 and 0.92, indicating high reproducibility (n=6 pairs). Analysis of the total protein expression profiles from the 3 sample groups (untreated, sms14-treated, smsdx-treated) revealed both similarities and differences. Figure 1 shows representative 2-DE gels of a negative control sample, a sms14- and a smsdx-treated sample.

Additionally, 489 protein spots were ≥2-fold down-regulated in smsdx-treated cells compared to negative control samples (Figure 2, p<0.05).

The intersections of the datasets, i.e. the changes between negative control versus sms14-treated and the changes between negative control versus smsdx-treated, were analysed. The set of 63 protein spots were ≥2-fold up-regulated in smsdx-treated cells compared to the negative control samples (p<0.05 Mann-Whitney analysis). On the other hand, 386 protein spots were ≥2-fold down-regulated in smsdx-treated cells compared to the negative control cells (Table I).

Protein expression, quantitative differences. Significant quantitative differences were observed in the protein expression patterns between the negative control cells and smsdx-treated cells. A set of 63 protein spots was ≥2-fold up-regulated in smsdx-treated cells compared to negative control samples (Figure 2, p<0.05).

The intersections of the datasets, i.e. the changes between negative control versus sms14-treated and the changes between negative control versus smsdx-treated, were analysed. The set of 63 protein spots were ≥2-fold up-regulated in smsdx-treated cells. Twenty-four (33.33%) of these were also present among the 46 protein spots that were up-regulated by more than 2-fold in sms14-treated cells. A total of 259 protein spots were common between the 489 and 386 that were ≥2-fold down-regulated in smsdx- and sms14-treated cells, respectively, compared to negative control cells. The overlap in the expressed proteins indicates a certain similarity between the sms14- and smsdx-treated cells.
Figure 1. Representative 2-DE maps derived from whole cell extracts of control, sms14- and smsdx-treated LNCaP cells (enlarged are gel segments showing similarities and significantly up-regulated spots in the 2 treated cells compared with positive control cells).

Figure 2. Hierarchical cluster analysis of 12 LNCaP control and treated samples. The dendrogram represents the expression patterns of 63 protein spots in 4 control (Blue), 4 sms14-treated (Red) and 4 smsdx-treated (Green) cells. The columns represent individual samples, while the rows represent the proteins. The colours in each cell unit correspond to the expression levels of a given protein in a particular sample (Red=up-regulation, Black=down-regulation).

Figure 3. Comparison of sms14 and smsdx samples. Cluster analysis of a set of 44 spots were >2-fold up-regulated in smsdx-treated cells compared to sms14-treated samples.
Differences in protein expression were determined by comparing the total number of differentially-expressed spots between smsdx- and sms14-treated cells. Quantitative differences were observed in the protein expression patterns between sms14- and smsdx-treated cells. A set of 44 protein spots was ≥2-fold up-regulated in smsdx-treated cells compared to sms14 samples. The difference is statistically significant using the Mann-Whitney signed-ranked test \( (p<0.05) \). On the contrary, 49 protein spots were ≥2-fold up-regulated in sms14-treated cell compared to smsdx-treated cells.

**Sample classification by hierarchical cluster analysis of differentially-expressed proteins.** The expressions of the set of 63 protein spots, ≥2-fold up-regulated in smsdx-treated cells compared to negative control samples, were subjected to cluster analysis (Figure 2).

All the samples were distinctively separated into 2 main branches on the dendrogram. The first branch consisted of all 4 control samples, while the second main branch was subdivided into 2 minor branches that separate sms14 from smsdx. Similarly, the 44 protein spots that were differentially-expressed by sms14-treated cells were subjected to hierarchical cluster analysis and classified (Figure 3).

In this analysis, both the negative control and smsdx samples were closely classified on the same main branch while the sms14 samples were on the second main branch.

**Protein expression, qualitative differences.** Approximately 1096 protein spots were separated by high resolution 2-DPAGE and 1005 of these spots could be matched between all the 12 gels. Eight protein spots were exclusively expressed in smsdx-treated cells compared to 5 in sms14-treated cells. These spots were very faint and a future analysis is warranted using narrow pH strips and loading more protein to the gels. The expressions of 736 proteins were unchanged between the negative control and smsdx samples and 676 were unchanged between the negative control and sms14 samples. Approximately 78% (551) of these proteins were expressed in the 736 and 676 datasets, i.e. smsdx and sms14 (Table II).

Approximately 50% of the total number of the resolved spots of smsdx-treated and sms14-treated cells had similar expression patterns compared to the negative control.

**Discussion**

The 2-DE technique has been used previously to visualize and subsequently analyse complex protein expressions in several human cancers (21, 22).

The presence of SSTR in the LNCaP cell line has been demonstrated by Dizeyi and co-workers (14). In previous studies, a number of proteins expressed by LNCaP were

---

**Table I. Quantitative differences of protein expression after sms14 and smsdx treatment.**

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Total valid spots</th>
<th>Matched spots (%)</th>
<th>&gt;2-fold up (*)</th>
<th>&gt;2-fold down</th>
<th>Sig. diff. ( (p&lt;0.005) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1155</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>sms14</td>
<td>1059</td>
<td>92</td>
<td>171</td>
<td>386 (295$)</td>
<td>46 (24**)</td>
</tr>
<tr>
<td>smsdx</td>
<td>1074</td>
<td>93</td>
<td>188</td>
<td>489(295$)</td>
<td>63 (24**)</td>
</tr>
</tbody>
</table>

*Comparison with control

$\$Intersection between 386 and 489 protein spots datasets

**Table II. Qualitative differences of protein expression after sms14 and smsdx treatment.**

<table>
<thead>
<tr>
<th>Qualitative changes</th>
<th>sms14</th>
<th>smsdx</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Total number of resolved spots</td>
<td>1059</td>
<td>1074</td>
</tr>
<tr>
<td>2 Fraction of matched spots</td>
<td>92%</td>
<td>93%</td>
</tr>
<tr>
<td>3 Total optical density</td>
<td>43453</td>
<td>49813</td>
</tr>
<tr>
<td>4 Correlation coefficient of pairs of control vs. sms14</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>5 Correlation coefficient of pairs of control vs. smsdx</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>6 Correlation coefficient of pairs of sms14 vs. smsdx</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>7 Number of conserved spots between control, sms14 and smsdx</td>
<td>551</td>
<td>551</td>
</tr>
<tr>
<td>8 Number of spots matched to all sms14 and smsdx samples</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>9 Unique spots</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>
found to be both up-regulated and down-regulated, as a result of treating the cell line with sms (23). Earlier in vitro data, by Brevini et al. (9), demonstrated that sms exerts a significant inhibition on the proliferation of LNCaP, due its effect on DNA synthesis. It has also been postulated that sms can exert an anti-proliferative effect on the LNCaP through an "autocrine/paracrine sms loop" (24). Proteomic analysis has recently been described as having the potential to improve prostate cancer diagnosis and to make possible the discovery of new prostate cancer biomarkers (25). In this study, the changes in protein expression induced in LNCaP cells on treatment with sms (sms14 and smsdx) were investigated. The study was an effort to increase the understanding of the mechanisms involved when treating with smsdx. The present results demonstrate that in vitro treatment with smsdx changes the proteome of LNCaP cells, as manifested by the up- and down-regulations of proteins. It is interesting to observe the similarities in effects between the native sms14 and the smsdx derivative. The differences may be explained by a certain effect of the carbohydrate moiety of the smsdx derivative.

Proteomic analysis can analyse, qualitatively and quantitatively, large expression profiles of thousands of polypeptides simultaneously. The mini 2-DE gel system used in this study resulted in protein separation with high quality resolution, that is comparable to the large 2-DE format. It further allows rapid screening of samples more efficiently and cost-effectively, thus making the application of 2-DE closer to daily clinical use.

Mini 2-DE was used to study the effect on the LNCaP proteome by incubating the cells with smsdx and sms14 for 3 days at 1 nM concentration (9). The treatment resulted in a decrease in the total number of resolved polypeptides. The identification of these protein spots will give information that may elucidate some pharmacological effects and cellular responses to smsdx treatment. The proteomic "profiling approach" used in this study allows analysis of multiple polypeptides simultaneously with the possibility of eventually identifying protein markers of importance involved in prostate cancer treatment using sms derivatives.

Identification of the affected proteins using protein mass spectrometry (MS) is in progress and will soon be reported.

Acknowledgements

Supported by grants from the Cancer Society in Stockholm, The King Gustav V Jubilee Fund, Stockholm and The Swedish Cancer Society.

References


Received October 20, 2005
Accepted October 31, 2005