International Journal of
Cancer Research

Protein Changes Typical for Therapy-resistant Cancer Cells Appear in MCF7 Breast Cancer Cultures as Early as One Doubling Time after Chemical Treatment

[1]Benjamin Y. Klein, [2]Souvenir D. Tachado, Henry Koziel and Hava K. Avraham
[1]Laboratory of Experimental Surgery, Hadassah University Hospital,
Ein-Kerem, Jerusalem 91120, Israel
[2]Beth-Israel Medical Center Division of Experimental Medicine, Beth Israel
Deaconess Medical Center and Harvard Medical School, Boston, MA 02115, USA

Abstract: Presumably any tumor cell population contains concomitantly, therapy-sensitive and therapy-resistant cells in contrast to the opinion, still held by many, that drug resistance develops only after repeated cycles of treatment. To test this hypothesis we selected a breast cancer cell line that under exposure to AGL2592 (Stat3 inhibitor) will result, along a wide concentration range, in steady levels of residual cell counts. MCF7 and T47D breast cancer cell lines showed a low ic_{50} (0.4 and 0.5 µM, respectively) from which we chose MCF7 because it showed only 10% residual cells compared to 40% in T47D. Proteomes of resistant and untreated MCF7 cells were run on 2D-gels for comparative analysis of protein expression. Calreticulin (CRT) and protein disulfide isomerase (PDI) showed lower abundance in treated cultures. Glycolytic enzymes, enolase1, aldolase A and glyceraldehydes 3 phosphate dehydrogenase (GAPDH) were more abundant and less modified in treated cultures. AGL2529-sensitivity was associated with part of the GAPDH molecules susceptible to cleavage at Ile^{170} and adenylation between Ile^{170} and Lys^{186}. The differential profile exhibited by all the above proteins is (according to others) the hallmark of unfavorable prognosis, which suggests that therapy resistant and sensitive MCF7 populations may coexist in this cell line in its pre-selected form.

Key words: Stat3, glycolytic enzymes, 2D-gel, mass-spectrometry, disulfide-isomerase, calreticulin

Introduction

Therapy resistance of relapsing cancer is one of the many unresolved issues in tumor cell biology. A frequent scenario in Breast Cancer (BC) hormonal and chemotherapy, is that an apparent BC eradication is followed by tumor-relapse that shows increased resistance to therapy (Tonetti and Jordan, 1995). *In vivo* BC cell populations initially respond to chemotherapy by increased apoptosis and cell proliferation, which decline within one month from the start of chemotherapy (Dowsett *et al.*, 1999) and are later followed by BC relapse. The cells responsible for BC relapse are chemo-resistant residual cells. Similarly, chemical treatment of BC cells in culture frequently results in residual chemo-resistant tumor cell populations remaining in culture after the death of the chemo-sensitive cells (Palmari *et al.*, 2000). Much of the effort in studying

---

chemo-resistance mechanisms has been focused on multidrug resistance molecules (MDR). However, MDR glycoproteins are not always active in drug resistant tumors (Salvatore *et al.*, 1999) and are not expected to play a role in irradiation-resistant tumor cells. This indicates that search for additional therapy-resistance mechanisms, is warranted. Cancer cells differ, from their normal counterparts, by reliance on the glycolytic metabolism similar to the strategy that normal cells adopt temporarily under anaerobic conditions. Increased consumption of glucose by tumors, while relinquishing the highly economic "benefits" of the Krebs cycle and oxygen-driven oxidative phosphorylation, has been noticed 80 years ago by Otto Warburg. The Warburg effect refers to the restricted use of glycolysis independently of oxygen presence and its fulfillment is associated with a change in the control of protein expression (Unwin *et al.*, 2003). Normally hypoxia inducible factor-1 (HIF-1) regulates angiogenic factors and glycolitic enzymes expression, in response to low oxygen levels. Examples where HIF-1 expression may be regulated in disregard of oxygen, are its induction when c-Src is over-expressed (Karni *et al.*, 2002) and by increased pyruvate synthesis (Lu *et al.*, 2002) that inhibits HIF-1 degradation. Thus, the Warburg effect distinguishes tumor from normal cells, nevertheless, our question is which protein changes distinguish therapy resistant from therapy sensitive BC cells? In the later case therapy at least distinguishes between the 2 roughly defined phenotypes. Being influenced by the tumor stem-cell hypothesis, our experimental model assumes the concomitant existence of chemo-resistant and chemo-sensitive cell populations (even in cultured BC cell lines and) regardless of the nature of anti cancer therapy. This model is inline with the concept of carcinogenegis in mammary stem-cells (Smalley and Ashworth, 2003). Hypothetically, stem-cells becoming tumors could proliferate continuously giving off progeny, half of which will lose "stem-ness" and become prone to apoptosis, i.e., becoming more sensitive to therapy, the rest would remain resistant stem-cells. Whether this turns out to be the case or not it seemed advisable to reveal differential proteomics in chemo-resistant cells. It is reasonable that chemo-resistant BC cells contain potential molecular targets for supplemental chemotherapeutic agent/s. Such putative targets would be difficult to discern by the use of comparative transcriptomics, because our current inability to predict post-translational modifications of proteins based on mRNA (cDNA) levels. This problem is also reflected by the difficulty to discern resistance from sensitivity by comparative transcriptomics in the different clinical stages in BC patients (Ma *et al.*, 2003). The hypothesis that the BC cell population contains cells that apriori are resistant to chemotherapy, (which is well fitting into the BC stem-cell hypothesis (Smalley and Ashworth, 2003)) required pilot experiments to test its validity and to suggest a cell-culture model. We chose to use two tyrphostins AG 1478 and AGL2592 to inhibit the growth of 2 breast cancer cell lines, MCF7 and T47D, both express low levels of EGFR (ErbB2) (Daly *et al.*, 1997) (target for AG1478). BC cells belong to a group of cancers that over-express Stat proteins (Real *et al.*, 2002; Zhang *et al.*, 2003), inhibition of Stat3 underscored the importance of its activity in BC (Li and Shaw, 2002). In normal breast epithelium 2 alternative activators of Stat3 were demonstrated, either by Jaks or by c-Src kinases, these were suggested to result in 2 different functional modules (Vultur *et al.*, 2004). Stat3 inhibition via Jaks (but not via Src) antagonized the cell adherence-induced Stat3 phosphorylation. However in the BC cells Stat3 constitutive activity was blocked via both, Jak and Src. AGL2592 was shown to inhibit Stat3 phosphorylation in leukemic (Ben-Bassat *et al.*, 2002) and in ovarian cancer cell lines (Arbel *et al.*, 2003). In the present work we chose to inhibit Stat3 based on the above information, but also because it is an inducer of several anti-apoptotic (survival) proteins like Bcl-2 and ERK 1/2 which we could follow in the cells that survived treatment. We show that an MCF7 cell population resistant to AGL2592 exists (apriori) within untreated cultures. This resistant MCF7 population is differentially expressing several proteins in a manner that is consistent with the different pattern of their expression in malignant/metastatic relative to less malignant or to normal cells (Table 2).

Materials and Methods

Reagents

Antibodies against phosphorylated dual specificity kinase target (pT-E-pY) and protease inhibitors were from Sigma, anti Bcl-2 antibodies were purchased from Sant-Cruze. MCF7 and T47D breast cancer cells were from ATCC. Cells were grown in Dulbecco's modified Eagle's medium 10% fetal calf serum 50 mg mL^{-1} penicillin and streptomycin (Invitrogen). Tyrphostins AG1478 and AGL2592 were a gift from Alexander Levitzki.

Cell Count and Determination of IC$_{50}$

Cells were plated at 3 or 5×10^3 cell/well in microtiter plates and after 24 h medium containing tyrphostins was added to final concentrations indicated in Fig. 1. The plates were fixed 48 h after exposure to the tyrphostins by adding 0.5% glutaraldehyde. The cultures were stained with 0.1% methylene blue in 0.1 M borate buffer pH 8.6 for 60 min. Surplus stain was thoroughly washed with distilled water and the residual stain was eluted with 0.1N Hcl and counted by an ELISA counter at 620 nm.

Electrophoresis and Western Blot Development

Cells were seeded, 10^4 cm^{-2}, in 25 cm^2 flasks, after 24 h medium with different concentrations of AGL2592 was applied to the cultures, 48 h later the cultures were harvested and subjected to cell lysis. Flasks were placed on ice and washed twice with 6 mL of cold PBS removing the washing solution to dryness. Ice-cold electrophoresis sample buffer (with 7% 2-mercaptoethanol and 3% sodium dodecylsulfate (SDS)) 100 µL/flask was added to incubate for 30 min on ice. The lysates were quickly scraped off the plastic with a policeman and transferred to cold tubes, for quick spinning, 2 min at 14,000 rpm, boiled for 5 min, cooled on ice and stored at -70°C until further use. Samples of lysed cultures representing equal protein concentrations and adjusted to equal volumes with plain sample buffer were subjected to polyacrylamide gel electrophoresis. Gels were then electroblotted onto PVDF filters that were blocked with 3% bovine serum albumin and 0.2% Tween 20. Filters were first incubated with anti Bcl-2 antibody and after washing they were incubated with anti rabbit IgG second antibody conjugated to peroxidase and the chemilluminescent was detected by autoradiography. Immunoblot results were analyzed by scanning of chemilluminescent autoradiographies and performing image analysis on the bands, using Scion-NIHimage software.

Comparative Proteomics

Comparative 2 dimensional electrophoresis of AGL2592 treated and control MCF7 breast cancer cells. MCF7 cells were seeded in 150 cm^2 culture flasks, treated with 10 µM AGL2592 or carrier and harvested on ice. The cell lysates where precipitated in cold 95% ethanol 5% de-ionized water overnight at -20°C The precipitate was washed in 90% ethanol 10% water and air-dried at -20°C. The protein pellets were dissolved in 300 µL of isoelectric focusing (IEF) sample buffer and 1.3 mg of each sample were adsorbed into 24 cm IPG strips (IEF at pH 3-10 range) by in-gel re-hydration at low voltage for 3 h. IEF was performed during 24 h by a total of 55000 volt-hours and equilibrated 15 min with the DTT and SDS-containing 2nd dimension sample buffer followed by 15 min equilibration with iodoacetamide. The samples of AGL2592 and control were ran concomitantly on 2 identical 10% polyacrylamide gels and stained with Coomassie blue. Protein spots that showed clear difference between the two 2D gels were removed and placed into acetonitrile until trypsin in-gel digestion was performed. After peptide extraction and desalting mass spectrometry for the specific proteins was done with collision induced dissociation of amino acid residues, detecting their time of flight on a Q-TOF-2 device. Proteins were identified with Mascot and Global-Server software.

Results

Selection of Chemo-therapy Compound

MCF7 and T47D breast cancer cells were exposed to decreasing concentrations of AGL2592 and AG1478, 24 h after they have been seeded in microtiter plates. The cultures were fixed and stained for counting 48 h after exposure to these compounds. Figure 1a shows that MCF7 cells are more sensitive to the EGFR kinase inhibitor (AG1478) with an IC_{50} of 17 µM then the T47D cells (IC_{50} = 27 µM, Fig. 1b). However, AGL2592 was a much more effective cytotoxic agent than AG1478 in both cell lines (4 and 5 µM, Fig 1a). Although the IC_{50} obtained for the Stat3 activation inhibitor AGL2592 was
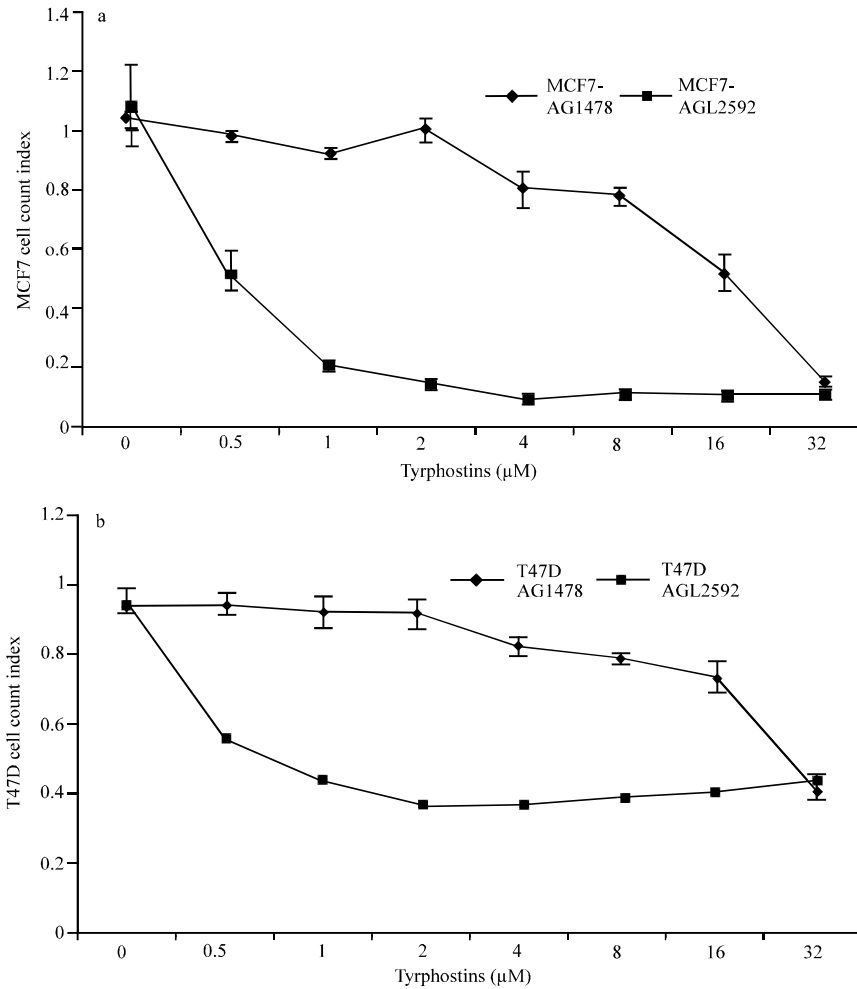

Fig. 1: Dose response of MCF7 and T47D, breast cancer cell lines, to tyrphostins. Cells were seeded at 5×10^3/well and 24 h later the medium was brought to indicated concentrations of the tyrphostins, a = AG1478, b = AGL2592. Fourty eight hours after exposure to these compounds, the cultures were fixed and stained with methylene blue for cell counting. Cell counts are expressed as indices relative to untreated wells (n = 10 per point)
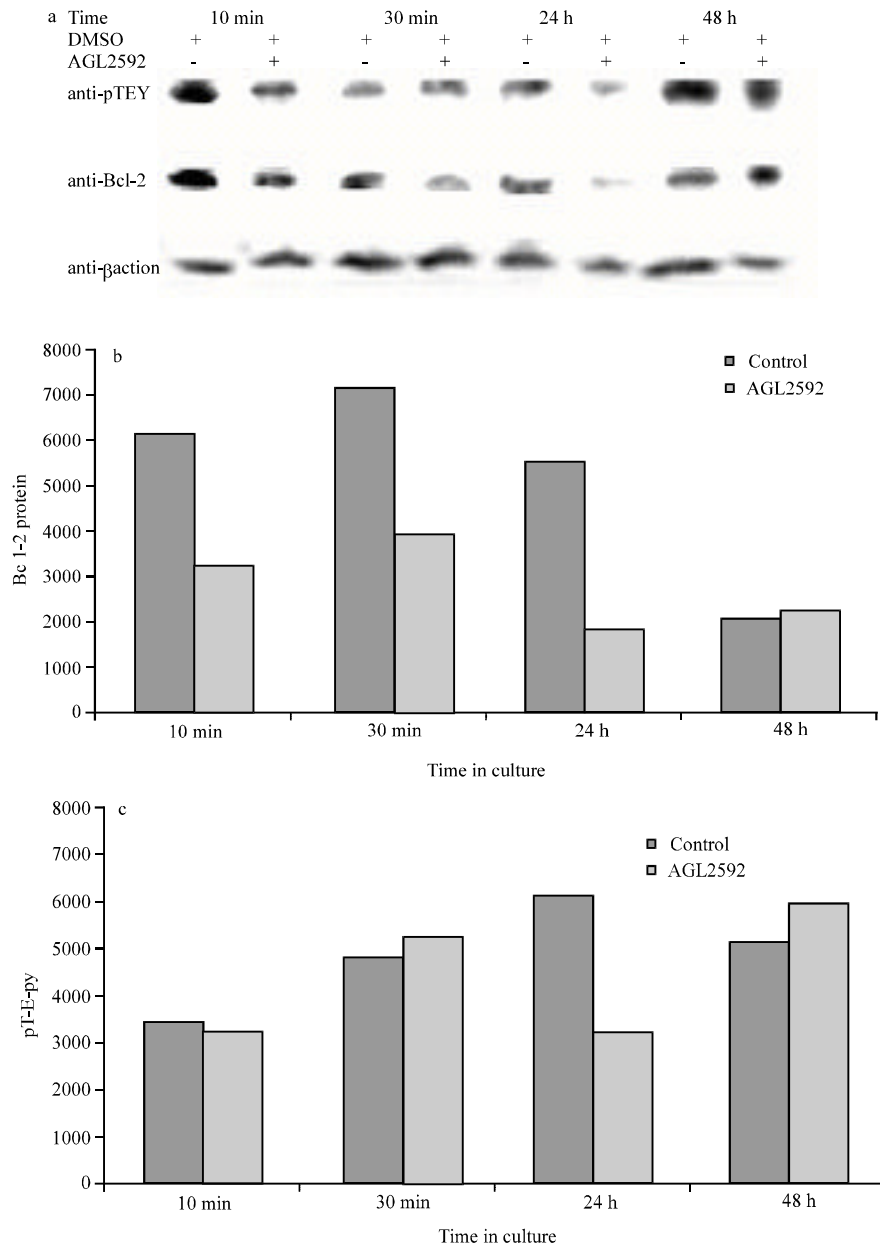
Fig. 2: Time course of MCF7 cells response of Bcl-2 and dual-specificity kinase (ERK1/2) motif under AGL2592. MCF7 cells were seeded, 10^6cells/25 cm^2 flask and 24 h later the medium was adjusted to 3 μM AGL2592 or the equivalent DMSO carrier concentration as controls. The cultures grew in the presence of the compound for indicated time intervals and then harvested with 100 μL RIPA on ice, 40 μg of protein equivalents were loaded on 10% SDS acrylamide gels. The Western blots were developed with indicated antibodies (a.) and the radiographic bands were scanned and their image (b = Bcl-2, c = Anti dual specificity kinase phospho-motif) was analyzed for quantitative density

in both cell lines (0.4 µM for MCF7 and 0.5 µM for T47D cells), the level of residual cell counts at 10 µM AGL2592 was fourfold higher in T47D cells (40%) than that of residual MCF7 cells (only10%). This indicates that T47D cells contain a fourfold larger chemo-resistant population than MCF7 cells. Therefore, if we were to compare the balk of untreated cells (that hypothetically) coexist with the resistant cells, then MCF7 residual cells are expected to differ from the untreated cells in a more pronounced manner and with a lower background than in the case of T47D cells. Hence, MCF7 cells seemed more suitable for a pilot study to explore proteomic markers of chemo-resistance.

Survival Protein in Resistant Cells

Anti-apoptotic proteins like Bcl-2 and ERK1/2 are Stat3-inducible and were followed as a marker to the AGL2592 effect. If cell death under AGL2592 in 90% of the MCF7 cells is accompanied, during the first exposure day, by a decrease in a survival protein e.g. Bcl-2, then 48 h later the 10% surviving cells should exhibit a Bcl-2 level that would be higher than the level in the dying cell population, (that can be observed only during the first 24 h). Indeed, Fig. 2 shows that at 48 h Bcl- 2 levels become much closer to the non-treated controls than seen after 10 min, 30 min and 24 h of exposure (Fig. 2a and b). Antibodies that specifically recognize the dual phosphorylation target characteristic of ERK1/2 show a steady level during the first 30 min (Fig. 2c), a low level after 24 h and a "recovery" after 48 h. This is consistent with a state in which the AGL2592-resistant population coexisted with the sensitive cell population before the treatment has been started, here the resistant cells reflect a straight forward selection of an existing, rather than a gradually developing, phenotype.

Differentially Exhibited Proteins in Agl2592-resistant Cells

The putative constant level of Bcl2 in residual MCF7 cells (relative to untreated) raised the following question: Is the sustained unchanged level of Bcl-2 (untreated Vs resistant cells at 48 h) in selected cells accompanied by proteomic dissimilarities between the chemo-resistant (residual) cells and the untreated cell population? To test this possibility, we treated MCF7 cells with 10 µM AGL2592 for 48 h and parallel neighboring cultures were treated with the DMSO carrier to serve as untreated controls. The extracted proteomes of AGL2592 and control cultures were run on a preparative two-dimensional (2D-gel) electrophoresis and the twin gels were stained with Coomassie blue. Fig. 3 shows a differential protein abundance in highly expressed (or presented) proteins that is prominent relatively to the background of many quantitativelly similar spots. We have picked some of the differentially expressed protein spots for identification by mass-spectrometry. Table 1 identifies the indicated protein spots. Calreticulin and PDI (disulfide isomerase) showed a lower abundance in the residual cells, 4.2 and 18 fold, respectively. Glyceraldehyde phosphate dehydrogenase (GAPDH), aldolase-A and enolase-1 showed in the residual cells a higher abundance than in the untreated controls, 1.97, 2.5 and 13.3 fold, respectively. In addition to the quantitative differences in protein abundance two remarkable phenomena can be observed, one relates to the appearance of bovine albumin in the gels and the other concerns the shift in the first dimension (IEF) mobility of aldolase-A and GAPDH. Several versions of BSA molecules (bovine serum albumin supplied through the fetal calf serum) appeared in a differential manner between both cell populations. In the residual cells the balk of BSA appeared mainly at its proper and expected relative-mobility (m_r) around 69 kDa, whereas in the gel of the untreated cells there was less BSA at the m_r of 69 kDa, however there was a substantial amount of BSA at lower m_r, possibly dimmers (140 kDa). In addition there were bands of BSA running in between the 69 and 140 kDa with a tendency to have a progressively slower mobility towards the acidic side of the 1st dimension. This kind of BSA bands is not observed in the residual cell gel. The gel of the residual cells showed in total 40% more albumin than that of the untreated cells.
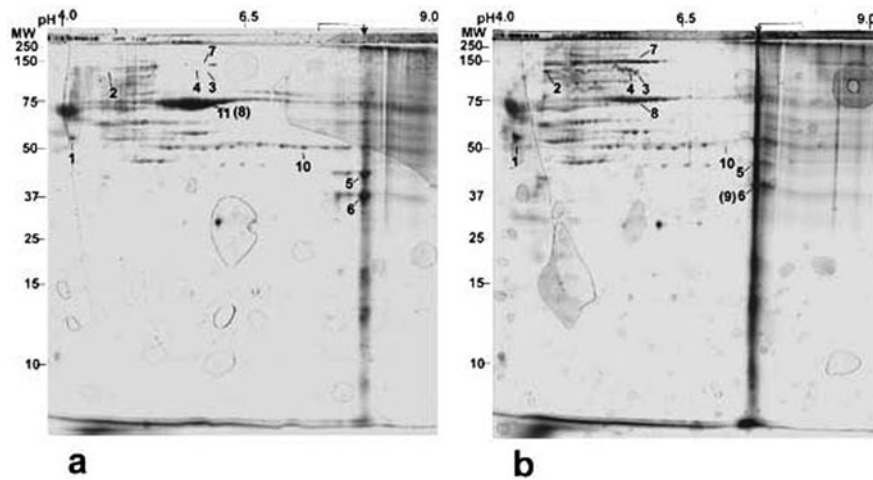
Fig. 3: Total proteomic 2D-gels, comparative between untreated and AGL2592-resistant MCF7 cells. MCF7 cells seeded 10^4 cells/cm^2 in 175 cm^2 flasks were exposed to 10 μM AGL2592 for 48 h and harvested on ice with RIPA in the presence of protease inhibitors. The cell lysates were precipitated in 95% ethanol at -20°C overnight and washed with 90% ethanol. The precipitates were suspended in IEF sample buffer and 1.3 mg protein from the treated and the untreated control cells and were run on 24 cm IEF strips with a pH range of 3-10. After equilibration with SDS/DTT and SDS/ iodoacetamide the 2nd dimension was run on 10% gels, fixed and stained with Coomassie blue. Left panel= AGL2592-treated residual cells, Right panel= untreated control cells (DMSO carrier). Arrows indicate enumerated spots identified in Table 1 that were picked for in-gel digestion, mass-spectrometry analysis and identification

Table 1: Differentially expressed protein spots in the comparative 2D-gels

Spot No.	Protein	pI	MW-kDa	Species	Score	Protein index # treated/untreated	Accession (gi-number)
1	Calreticulin	4.29	48.112	Human	573	0.236	4757900
2	PDI	4.77	57.059	Human	132	0.055	35655
3	BSA	5.82	69.248	Bovine	874	*	1351907
4	BSA	5.82	69.248	Bovine	244	*	1351907
5	Aldolase A	8.39	39.264	Human	363	1.97	229674
5a	GAPDH	8.26	36.031	Human	335	**	31645
6	GAPDH	8.26	36.031	Hhuman	943	2.5 normal Mr	31645
7	BSA	5.82	69.248	Bovine	727	0.105##	1351907
8	BSA	5.78	69.225	Bovine	512	2.5	418694
9	GAPDH	8.26	36.031	Human	613	0.4 (pH)	31645
10	Enolase 1	7.01	47.139	Hhuman	739	13.3	4503571
11	BSA	5.82	69.248	Bovine	1512	1.0 normal Mr	1351907

Arbitrary density units, ## BSA with relative mobility of dimmers, * BSA with intermediate mobility, **GAPDH adjacent to aldolase

The second remarkable phenomenon is the shift in aldolase-A and GAPDH to the acidic side in the untreated cell population. It should be noted that in both gels these two enzymes moved together on the IEF and were separated only during the 2nd dimension run, after equilibration with SDS+DTT followed by iodoacetamide treatment. The co-migration in the first dimension was seen also in the T47D gels (not shown). The difference in GAPDH abundance between the residual and control MCF7
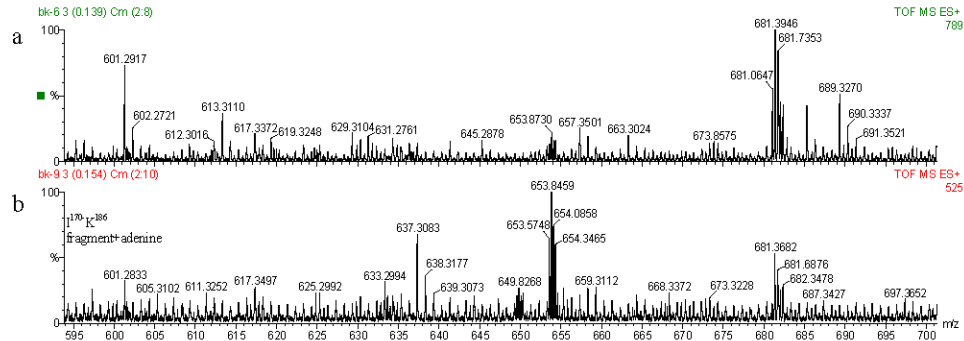
Fig. 4: Mass spectra comparison between glycerophoaphate dehydrogenase (GAPDH) protein spots. Panel a. shows the spectrum of GAPDH of AGL2592-resistant cells gel. Panel b. shows mass spectrum of untreated control cells. The Arrow indicates the broken and adenylated fragment, I^{170}-K^{186}

Table 2: Differential expression in tumor cells of proteins mentioned in Table 1

Protein	Cell or tissue	Normal/cancer, malignant/less malignant	Ref
CRT, PDI	Ductal Braest Cancer	Down	(Chahed *et al.*, 2005)
CRT	Normal Vs Necrotic MCF7	Low	(Poliseno *et al.*, 2004)
CRT	Neuroblastoma with bad/good prognosis	Down	(Hsu *et al.*, 2005)
CRT, PDI	Ductal Ca	Down	(Bini *et al.*, 1997)
CRT	Undifferentiated prostat	Down	(Ruddat *et al.*, 2005)
CRT	Colorectal	Down	(Alfonso *et al.*, 2005)
CRT	Colorectal with stable microsatellites/unstable	Low	(Banerjea *et al.*, 2004)
CRT	High/low metastatic hepatocellular carcinoma	Low	(Ding *et al.*, 2004)
CRT, PDI	IR-resistant prostate cancer	Down	(Prasad *et al.*, 1999)
GAPDH	Breast cancer	Up	(Lu *et al.*, 2002)
GAPDH	Resistance MCF7, agressive breast cancer	Up	(Revillion *et al.*, 2000)
Enolase	Breast cancer (Bcl-XL expressing)	Up	(Espana *et al.*, 2005)
Enolase	Breast cnacer (Her-2/neu expressing)	Up	(Zhang *et al.*, 2005)
Enolase	Infiltrating ductal carcinoma	Up	(Somiari *et al.*, 2003)
Aldolase	Rat breast cancer	Up	(Perumal *et al.*, 2005)
Aldolase	MCF7 under anoxia	Up	(Ameri *et al.*, 2002)
Aldolase, enolase	Breast cancerVs normal	Up	(Hennipman*et al.*, 1987)

populations was only 1.97 fold in favor of the residual cells. However, a fraction of the GAPDH molecules in the control cells was cleaved in an illegitimate residue (isoleucine[170]) that is not due to the trypsin in-gel treatment (Fig. 4). This residue is in midst of the catalytic domain of the GAPDH enzyme for which the MS analysis revealed an additional mass of 134 Daltons attached to the Ile[170]-K[186] residues fragment. The additional 134 Daltons are identified as adenine that must be covalently bound to Ile[170]-K[186] fragments (Fig. 4b), which were absent from the canonical GAPDH digest in the AGL2592-resistant cell population. This may constitute an added effect to the 1.97-fold abundance of GAPDH in favor of the residual cells because it might impede the normal enzyme activity in the control (sensitive) cells, for example in a negative dominant manner.

Table 2 shows that all the 5 proteins that were differentially expressed in the residual cells relative to the untreated cells are also known to be differentially expressed in malignant cells relative to their normal or less malignant counterparts. This indicates that after a minimal selection time of only one cell division (48 h) the pattern of differentially expressed proteins most probably belongs to cells that coexisted with the general MCF7 population and were simply selected by AGL2592.

Discussion

AGL2592 is a bistyrphostin (Ben-Bassat *et al.*, 2002) that has been shown to inhibit stat3 phosphorylation, perhaps by virtue of interference with dimerization of each of two putative Stat3 molecules on their way to become activated. The efficiency of growth arrest and cell death induction in breast cancer cells by this compound, relative to AG1478 (EGFR inhibitor) is consistent with the claims (Garcia *et al.*, 1997) that Stat3 is an important mediator of proliferation in breast and other cancer types (Catlett-Falcone *et al.*, 1999). The low proportion (10%) of residual cells under AGL2592, that were seen in MCF7 cultures made this cell line a suitable candidate for proteomic comparisons exploiting this case in which resistant cells coexist with the sensitive population. This is simply because the lower the resistant cell percentage the less prominent will be the contamination effect of its differentially expressed proteins on the negative control of the untreated population. Stat3 can activate transcription of several anti-apoptotic proteins like Bcl-2, Bcl-X, survival proteins and ERKs (Alas and Bonavida, 2003). Therefore, inhibition of Stat3 is consistent with a lower Bcl-2 abundance (relatively to controls) during the first 24 h interval that is included in the doubling time of the first cell-generation. Beyond this first cell doubling time (48 h) the Bcl-2 in the residual cells equalized with that of the untreated total population that reflects stability in this protein level and supports our hypothesis of a selection effect. The dual specificity-kinase target, the Thr-Glu-Tyr (TEY for short) is a relatively uncommon motif among proteins, in addition to ERK1/2 it is found in the metabotropic receptor of CNS cells and in Bcl-2 (T^{178}-E-Y^{180}). This motif is thus associated with these anti-apoptotic proteins and its decreased phosphorylated version at the 24 h interval in MCF7 cells and the "recovery" after 48 h may be well suited for the survival of the residual cells. There must be a specific mechanism (probably other than the multidrug-resistance glycoprotein) that enables some cells to ignore the AGL2592 potential to inhibit Bcl-2 expression and TEY motif phosphorylation. In spite of the interest one may find in the effect of AGL2592 on apoptotic signaling pathways, its importance for the present work is only marginal. The main purpose of the present work is to show that the expression pattern of various proteins associated with tumor resistance to therapy may be present in a restricted population of cells as early as one cell division after the therapy starts. This hard core of resistant cells may very well coexist with the sensitive cells even before the start of therapy.

With the hypothesis of coexistence of therapy sensitive and resistant cell populations in mind we looked at the Coomassie blue detectable proteins in differential 2D-gels. The most striking differences between the gels turned out to be the various versions of bovine serum albumin (BSA) that one would normally dismiss as a culture artifact derived from the fetal calf serum. However, the striking differences between the mobility in several BSA molecular species in the untreated MCF7 cells in contrast with their absence in the resistant cell gels, deserves a different logic. Based on the longstanding clinical information on serum albumin in cancer one could refer to BSA as a surrogate albumin replacing in culture the human serum albumin (HSA).

Serum albumin deficiency (inversion of albumin/globulin ratio) is a reliable marker for unfavorable prognosis in individuals suffering from one of several malignant tumors (Lis *et al.*, 2003) e.g., breast cancer. Albumin, synthesized mainly in the liver, is the most abundant serum protein; it is functioning as an oncotic-pressure protein by keeping a proper intra-vascular volume. Albumin binds lipids, fatty acids, ingested chemicals and a variety of small compounds, perhaps serving as a buffer or carrier of

drugs to peripheral tissues. Albumin can cross (*in vivo*) the vascular wall into the Extra Cellular Fluid (ECF) compartment across endothelial cells by a process known as transcytosis (Schnitzer, 1992) which is thought to be mediated by cell surface albumin-binding proteins. These binding proteins are considered to be albumin receptors, i.e., gp60 (albondin) (Schnitzer *et al.*, 1988), osteonectin (SPARK), gp18 and gp30 (Schnitzer and Oh, 1992). Albumin is a target for modifications (in addition to phosphorylation), it may undergo oxidation of its SH residues (Havea *et al.*, 2000) to form adducts and aggregates, albumin dimers may result also from reaction with hydroxyl radicals via generation of intermolecular bityrosins (Davies and Delsignore, 1987), nitration is also a possible modification (Greenacre *et al.*, 1999; Koeck *et al.*, 2004). It has been suggested that non-modified albumin is more avidly bound by gp60, in contrast, albumin modification by radicals increases its affinity to gp30 and gp18 (Schnitzer *et al.*, 1992) and makes it more susceptible to breakdown by proteases (Davies *et al.*, 1987). Aggregated and adduct albumin molecules can be distinguished by SDS-PAGE according to their mobility that differs from the unmodified molecules, these can be better exhibited in 2D gels (Havea *et al.*, 2000, 2001) and picked for mass spectrometry analysis. Recently it has been shown that several albumin receptors belong to the nuclear heterogeneous ribonuclear proteins (hnRNP) family (Fritzsche *et al.*, 2004) such as hnRNP A2/B1 which are known to increase in tumor cells (Satoh *et al.*, 2000) and were identified as the above mentioned gp30 and gp18, respectively. In addition calreticulin (CRT) was also found to bind albumin, this raises the possibility that the untreated MCF7 cells with higher CRT expression were more efficient in removing the unmodified albumin, perhaps explaining their lower level in untreated cells. Significantly, the resistant cells were more efficient in removing the modified slow migrating albumin species and less so in removing the normally migrating molecules. CRT, in addition to its act as an albumin binding protein (Fritzsche *et al.*, 2004), may interact with glucocorticoid and androgen hormone receptors. By this activity it could influence the function of these receptors in BC cells especially in MCF7 cells that are known as a sex hormone responsive model. CRT has been recently shown to inhibit the expression of GLUT-1 (glucose transporter-1) by negative regulation at the un-translated (UTR) GLUT-1 mRNA level(Totary-Jain *et al.*, 2005). This may explain part of the metabolic aspect of the resistance in the residual cells that due to lower CRT might express GLUT-1 more abundantly (for a high glucose uptake) which would support the glycolytic energy metabolism, a known advantage in malignant cells.

Protein Disulfide Isomerase (PDI) that has a chaperon effect by regulation of protein structure around disulfide bonds, among others it is important as a member of a tetramer with á-Peptidyl-prolyl hydroxylase (α-PPH) for protection and efficient secretion of collagen. Therefore a lower expression of PDI may be interpreted as a lesser dependence of the therapy resistant cells on extra cellular matrix collagen with an increased propensity in detachment to metastasize. PDI may perhaps interact as a chaperon with β-PPH, which senses levels of oxygen controlling HIF-1 turnover. Lower expression of PDI in the therapy resistant cells is consistent with upgrading of the Warburg effect in which the tumor cells can prevail in much lower oxygen levels and may afford decreased sensitivity to changes in oxygenation.

Aldolase-A a glycolytic enzyme has been shown to appear in metastasis of BC cells. In the gel of the sensitive cells it has moved almost one pH unit to the acidic side, indicating a possible modification.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), another glycolytic enzyme that ran toward the acidic side, in the gel of the pretreated sensitive cells, perhaps due to modification. The

Sequence No. enzyme	Consensus
↓	↓
1) 15-Hydroxy prostaglandin-dehydrogenase (human)	^{32}VALED
2) Lactate-dehydrogenase (procine)	^{48}VALED
3) Glutamate-dehydrogenase (bovine)	^{270}VAVGE
4) Hydroxy steroid dehydrogenase (streptomyces)	^{33}VVLAD
5) Ribitol-2-dehydrogenase (klesiella)	^{33}VVLID
6) GAPDH (human)	^{170}IVEGLMTTV AI tATQK
7) Adenylate kinase (chicken muscle)	^{28}KIVh KIVh ^{157}ATE ^{57}KLqAI
8) Isocidtrate-dehydrogenase (mitochondria)	^{121}LVpGWt a p I TIGr

Fig. 5: Consensus sequences of adenine ring-binding enzymes aligned with MCF7 GAPDH I^{170}-K^{186} fragment. Consensus sequences 1-5 and 7-8 represent Adenylate-photoaffinity-protected sequences of enzymatic digests as shown in several publications (Chavan *et al.*, 1993; Salvucci *et al.*, 1992; Sankaran *et al.*, 1996). The marked residues V^{178}, A^{180}, I^{181}, conform to the adenine-ring-binding consensus of aligned enzymes according to polar/non-polar amino acid classification. The low-case residues do not conform to the aligned motifs

adenine-conjugate in the I^{170}-K^{186} fragment that was seen in the therapy sensitive cell population may reflect part of the post translational modifications responsible for the shift in first dimension migration of the putative complex GAPDH/aldolase. It is difficult to argue that the adenine molecule is a residue broken off the NAD$^+$ coenzyme normally utilized by the GAPDH, because according to electron density analysis (Cowan-Jacob *et al.*, 2003) the adenine residue of NAD$^+$ has affinity to amino acids close to but still excluded from this fragment. The nicotinic moiety of NAD is bound to Cys^{149} (Cys^{152} in human GAPDH) on the N-terminal side of I^{170} and the adenine moiety of NAD may be in contact with His^{179} and other residues much closer to the C-terminus of GAPDH. In a monomeric form Cys^{152} and His^{179} are potentially bound by a hydrogen bond (Nagradova, 2001). It is not clear whether NAD or hydrogen bonding could "staple" together temporarily 2 cleavage products (cleaved at I^{170}) to enable their co-migration with intact GAPDH monomer molecules on the 2nd dimension under electrophoretic strain. GAPDH can fulfill functions unrelated to glycolysis and appear in forms of oligomeric structures, it may be active in membrane fusion, DNA repair and replication, RNA transport, apoptosis and microtubule bundling (Sirover, 1999). Its DNA repair capacity could be reflected in adenine binding of the above described fragment similar to canonical DNA ligases that utilize a lysine residue as an adenylation site for their activity, which makes such an adenylated lysine resistant to trypsin cleavage (Tomkinson *et al.*, 1991). However, the I^{170}-K^{186} fragment doesn't contain a trypsin un-cleaved lysine-end, therefore the adenine is probably bound to a residue other than lysine. In Fig. 5 this GAPDH-derived I^{170}-K^{186} fragment is aligned with consensus sequences that were found to be adenine-ring binding motifs. Motifs 1-5 (Chavan *et al.*, 1993) and motifs at No.8 (Sankaran *et al.*, 1996) are derived from hydrogenases and motifs at No. 7 (Salvucci *et al.*, 1992) are from adenylate kinase. According to this alignment the three marked residues in this GAPDH fragment may bind a dephosphorylated adenine-ring. Poly (ADP-ribose) polymerase (PARP) can bind and inhibit the activity of oxidation-modified GAPDH (Du *et al.*, 2003), it might underlay the adenylation of the I^{170}-K^{186} fragment.

Note that the microtubule bundling ability of GAPDH may reflect its co-migration with aldolase A during IEF. These two enzymes may be non-covalently bound to each other and to cytoskeleton components (Volker *et al.*, 1995) to form microtubules networks as an enzyme complex, supporting their catalytic activities on their consecutive substrate/product targets.

Enolase 1 (phosphopyruvate hydratase) appeared at its expected isoelectric point in the therapy-resistant cells and it does not appear in the respective spot of the untreated cell population. Taken together, the relative abundance of these glycolytic enzymes in the AGL2592-treatment residual cells indicate that part of the proteomic basis for therapy resistance in MCF7 cells is associated with increased intensity of the Warburg effect, or the ability of this effect to persist under the therapeutic stress.

Acknowledgement

References

Alas, S. and B. Bonavida, 2003. Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. Clin. Cancer Res., 9: 316-326.

Alfonso, P., A. Nunez, J. Madoz-Gurpide, L. Lombardia, L. Sanchez and J.I. Casal, 2005. Proteomic expression analysis of colorectal cancer by two-dimensional differential gel electrophoresis. Proteomics, 5: 2602-2611.

Ameri, K., B. Burke, C.E. Lewis and A.L. Harris, 2002. Regulation of a rat VL30 element in human breast cancer cells in hypoxia and anoxia: Role of HIF-1. Br. J. Cancer, 87: 1173-1181.

Arbel, R., N. Rojansky, B.Y. Klein, R. Levitzki, Z. Hartzstark, N. Laufer and H. Ben-Bassat, 2003. Inhibitors that target protein kinases for the treatment of ovarian carcinoma. Am. J. Obstet. Gynecol., 188: 1283-1290.

Banerjea, A., S. Ahmed, R.E. Hands, F. Huang, X. Han, P.M. Shaw, R. Feakins, S.A. Bustin and S. Dorudi, 2004. Colorectal cancers with microsatellite instability display mRNA expression signatures characteristic of increased immunogenicity. Mol. Cancer, 3: 21.

Ben-Bassat, H., Z. Hartzstark, R. Levitzki, B.Y. Klein, Z. Shlomai, A. Gazit and A. Levitzki, 2002. Tyrosine kinase inhibitors suppress the growth of non-hodgkin B lymphomas. J. Pharmacol. Exp. Ther., 303: 163-171.

Bini, L., B. Magi, B. Marzocchi, F. Arcuri, S. Tripodi, M. Cintorino, J.C. Sanchez, S. Frutiger, G. Hughes and V. Pallini *et al.*, 1997. Protein expression profiles in human breast ductal carcinoma and histologically normal tissue. Electrophoresis, 18: 2832-2841.

Catlett-Falcone, R., W.S. Dalton and R. Jove, 1999. STAT proteins as novel targets for cancer therapy. Signal transducer an activator of transcription. Curr. Opin Oncol., 11: 490-496.

Chahed, K., M. Kabbage, L. Ehret-Sabatier, C. Lemaitre-Guillier, S. Remadi, J. Hoebeke and L. Chouchane, 2005. Expression of fibrinogen E-fragment and fibrin E-fragment is inhibited in the human infiltrating ductal carcinoma of the breast: The two-dimensional electrophoresis and MALDI-TOF-mass spectrometry analyses. Intl. J. Oncol., 27: 1425-1431.

Chavan, A.J., C.M. Ensor, P. Wu, B.E. Haley and H.H. Tai, 1993. Photoaffinity labeling of human placental NAD(+)-linked 15-hydroxyprostaglandin dehydrogenase with (alpha-32P)2N3NAD+. Identification of a peptide in the adenine ring binding domain. J. Biol. Chem., 268: 16437-16442.

Cowan-Jacob, S.W., M. Kaufmann, A.N. Anselmo, W. Stark and M.G. Grutter, 2003. Structure of rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase. Acta Crystallogr. D Biol. Crystallogr., 59: 2218-2227.

Daly, J.M., C.B. Jannot, R.R. Beerli, D. Graus-Porta, F.G. Maurer and N.E. Hynes, 1997. Neu differentiation factor induces ErbB2 down-regulation and apoptosis of ErbB2-overexpressing breast tumor cells. Cancer Res., 57: 3804-3811.

Davies, K.J. and M.E. Delsignore, 1987. Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. J. Biol .Chem., 262: 9908-9913.

Davies, K.J., M.E. Delsignore and S.W. Lin, 1987. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J. Biol. Chem., 262: 9902-9907.

Ding, S.J., Y. Li, X.X. Shao, H. Zhou, R. Zeng, Z.Y. Tang and Q.C. Xia, 2004. Proteome analysis of hepatocellular carcinoma cell strains, MHCC97-H and MHCC97-L, with different metastasis potentials. Proteomics, 4: 982-994.

Dowsett, M., C. Archer, L. Assersohn, R.K. Gregory, P.A. Ellis, J. Salter, J. Chang, P. Mainwaring, I. Boeddinghaus and S.R. Johnston *et al.*, 1999. Clinical studies of apoptosis and proliferation in breast cancer. Endocr. Relat. Cancer, 6: 25-28.

Du, X., T. Matsumura, D. Edelstein, L. Rossetti, Z. Zsengeller, C. Szabo and M. Brownlee, 2003. Inhibition of GAPDH activity by poly (ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. J. Clin. Invest., 112: 1049-1057.

Espana, L., B. Martin, R. Aragues, C. Chiva, B. Oliva D. Andreu and A. Sierra, 2005. Bcl-x (L)-mediated changes in metabolic pathways of breast cancer cells: From survival in the blood stream to organ-specific metastasis. Am. J. Pathol., 167: 1125-1137.

Fritzsche, T., M. Schnolzer, S. Fiedler, M. Weigand, M. Wiessler and E. Frei, 2004. Isolation and identification of heterogeneous nuclear ribonucleoproteins (hnRNP) from purified plasma membranes of human tumour cell lines as albumin-binding proteins. Biochem. Pharmacol., 67: 655-665.

Garcia, R., C.L. Yu, A. Hudnall, R. Catlett, K.L. Nelson, T. Smithgall, D.J. Fujita, S.P. Ethier and R. Jove. 1997. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. Cell Growth Differ, 8: 1267-1276.

Greenacre, S.A., P. Evans, B. Halliwell and S.D. Brain, 1999. Formation and loss of nitrated proteins in peroxynitrite-treated rat skin *in vivo*. Biochem. Biophys. Res. Commun., 262: 781-786.

Havea, P., H. Singh and L.K. Creamer, 2000. Formation of new protein structures in heated mixtures of BSA and alpha-lactalbumin. J. Agric. Food Chem., 48: 1548-1556.

Havea, P., H. Singh and L.K. Creamer, 2001. Characterization of heat-induced aggregates of beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin in a whey protein concentrate environment. J. Dairy Res., 68: 483-497.

Hennipman, A., J. Smits, B. van Oirschot, J.C. van Houwelingen, G. Rijksen, J.P. Neyt, J.A. Van Unnik and G.E. Staal, 1987. Glycolytic enzymes in breast cancer, benign breast disease and normal breast tissue. Tumour Biol., 8: 251-263.

Hsu, W.M., F.J. Hsieh, Y.M. Jeng, M.L. Kuo, C.N. Chen, D.M. Lai, L.J. Hsieh, B.T. Wang, P.N. Tsao and H. Lee *et al.*, 2005. Calreticulin expression in neuroblastoma--a novel independent prognostic factor. Ann. Oncol., 16: 314-321.

Karni, R., Y. Dor, E. Keshet, O. Meyuhas and A. Levitzki, 2002. Activated pp60c-Src leads to elevated hypoxia-inducible factor (HIF)-1alpha expression under normoxia. J. Biol. Chem., 277: 42919-42925.

Koeck, T., B. Levison, S.L. Hazen, J.W. Crabb, D.J. Stuehr and K.S. Aulak, 2004. Tyrosine nitration impairs Mammalian aldolase a activity. Mol. Cell Proteomics, 3: 548-557.

Li, L. and P.E. Shaw, 2002. Autocrine-mediated activation of STAT3 correlates with cell proliferation in breast carcinoma lines. J. Biol. Chem., 277: 17397-17405.

Lis, C.G., J.F. Grutsch, P.G. Vashi and C.A. Lammersfeld, 2003. Is serum albumin an independent predictor of survival in patients with breast cancer? JPEN J. Parenter. Enteral Nutr., 27: 10-15.

Lu, H., Y. Zhang, D.D. Roberts, C.K. Osborne and N.S. Templeton, 2002. Enhanced gene expression in breast cancer cells *in vitro* and tumors *in vivo*. Mol. Ther., 6: 783-792.

Ma, X.J., R. Salunga, J.T. Tuggle, J. Gaudet, E. Enright, P. McQuary, T. Payette, M. Pistone, K. Stecker and B.M. Zhang *et al.*, 2003. Gene expression profiles of human breast cancer progression. Proc. Natl. Acad. Sci. USA, 100: 5974-5979.

Nagradova, N.K., 2001. Study of the properties of phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase. Biochemistry (Mosc), 66: 1067-1076.

Palmari, J., F. Wallet, J. Berard, Y. Berthois, P.M. Martin and C. Dussert, 2000. Morphological evidence for a subpopulation selection effect by estrogen and antiestrogen treatments in the heterogeneous MCF-7 cell line. Anal. Cell Pathol., 20: 99-113.

Perumal, S.S., P. Shanthi and P. Sachdanandam, 2005. Therapeutic effect of tamoxifen and energy-modulating vitamins on carbohydrate-metabolizing enzymes in breast cancer. Cancer Chemother. Pharmacol., 56: 105-114.

Poliseno, L., L. Bianchi, L. Citti, S. Liberatori, L. Mariani, A. Salvetti, M. Evangelista, L. Bini, V. Pallini and G. Rainaldi, 2004. Bcl2-low-expressing MCF7 cells undergo necrosis rather than apoptosis upon staurosporine treatment. Biochem. J., 379: 823-832.

Prasad, S.C., V.A. Soldatenkov, M.R. Kuettel, P.J. Thraves, X. Zou and A. Dritschilo, 1999. Protein changes associated with ionizing radiation-induced apoptosis in human prostate epithelial tumor cells. Electrophoresis, 20: 1065-1074.

Real, P.J., A. Sierra, A. De Juan, J.C. Segovia, J.M. Lopez-Vega and J.L. Fernandez-Luna, 2002. Resistance to chemotherapy via Stat3-dependent overexpression of Bcl-2 in metastatic breast cancer cells. Oncogene, 21: 7611-7618.

Revillion, F., V. Pawlowski, L. Hornez and J.P. Peyrat, 2000. Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. Eur. J. Cancer, 36: 1038-1042.

Ruddat, V.C., S. Whitman, R.D. Klein, S.M. Fischer and T.R. Holman, 2005. Evidence for downregulation of calcium signaling proteins in advanced mouse adenocarcinoma. Prostate, 64: 128-138.

Salvatore, C., M. Bigioni, E.M. Iafrate, M. Cianfriglia and S. Manzini, 1999. FCE 24517-resistant MCF-7 human breast cancer cell line: Selection and characterization. Anticancer Drugs, 10: 663-669.

Salvucci, M.E., A.J. Chavan and B.E. Haley, 1992. Identification of peptides from the adenine binding domains of ATP and AMP in adenylate kinase: Isolation of photoaffinity-labeled peptides by metal chelate chromatography. Biochemistry, 31: 4479-4487.

Sankaran, B., A.J. Chavan and B.E. Haley, 1996. Identification of adenine binding domain peptides of the NADP+ active site within porcine heart NADP(+)-dependent isocitrate dehydrogenase. Biochemistry, 35: 13501-13510.

Satoh, H., H. Kamma, H. Ishikawa, H. Horiguchi, M. Fujiwara, Y.T. Yamashita, M. Ohtsuka and K. Sekizawa, 2000. Expression of hnRNP A2/B1 proteins in human cancer cell lines. Intl. J. Oncol., 16: 763-767.

Schnitzer, J.E., 1992. gp60 is an albumin-binding glycoprotein expressed by continuous endothelium involved in albumin transcytosis. Am. J. Physiol., 262: H246-254.

Schnitzer, J.E., W.W. Carley and G.E. Palade, 1988. Albumin interacts specifically with a 60-kDa microvascular endothelial glycoprotein. Proc. Natl. Acad. Sci. USA, 85: 6773-6777.

Schnitzer, J.E. and P. Oh, 1992. Antibodies to SPARC inhibit albumin binding to SPARC, gp60 and microvascular endothelium. Am. J. Physiol., 263: H1872-1879.

Schnitzer, J.E., A. Sung, R. Horvat and J. Bravo, 1992. Preferential interaction of albumin-binding proteins, gp30 and gp18, with conformationally modified albumins. Presence in many cells and tissues with a possible role in catabolism. J. Biol. Chem., 267: 24544-24553.

Sirover, M.A., 1999. New insights into an old protein: The functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim. Biophys. Acta, 1432: 159-184.

Smalley, M. and A. Ashworth, 2003. Stem cells and breast cancer: A field in transit. Nat. Rev. Cancer, 3: 832-844.

Somiari, R.I., A. Sullivan, S. Russell, S. Somiari, H. Hu, R. Jordan, A. George, R. Katenhusen, A. Buchowiecka and C. Arciero *et al.*, 2003. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. Proteomics, 3: 1863-1873.

Tomkinson, A.E., N.F. Totty, M. Ginsburg and T. Lindahl, 1991. Location of the active site for enzyme-adenylate formation in DNA ligases. Proc. Natl. Acad. Sci. USA, 88: 400-404.

Tonetti, D.A. and V.C. Jordan, 1995. Possible mechanisms in the emergence of tamoxifen-resistant breast cancer. Anticancer Drugs, 6: 498-507.

Totary-Jain, H., T. Naveh-Many, Y. Riahi, N. Kaiser, J. Eckel and S. Sasson, 2005. Calreticulin destabilizes glucose transporter-1 mRNA in vascular endothelial and smooth muscle cells under high-glucose conditions. Circ. Res., 97: 1001-1008.

Unwin, R.D., R.A. Craven, P. Harnden, S. Hanrahan, N. Totty, M. Knowles, I. Eardley, P.J. Selby and R.E. Banks, 2003. Proteomic changes in renal cancer and co-ordinate demonstration of both the glycolytic and mitochondrial aspects of the Warburg effect. Proteomics, 3: 1620-1632.

Volker, K.W., C.A. Reinitz and H.R. Knull, 1995. Glycolytic enzymes and assembly of microtubule networks. Comp. Biochem. Physiol. B. Biochem. Mol. Biol., 112: 503-514.

Vultur, A., J. Cao, R. Arulanandam, J. Turkson, R. Jove, P. Greer, A. Craig, B. Elliott and L. Raptis, 2004. Cell-to-cell adhesion modulates Stat3 activity in normal and breast carcinoma cells. Oncogene.

Zhang, D., L.K. Tai, L.L. Wong, L.L. Chiu, S.K. Sethi and E.S. Koay, 2005. Proteomic Study Reveals That Proteins Involved in Metabolic and Detoxification Pathways Are Highly Expressed in HER-2/neu-positive Breast Cancer. Mol. Cell Proteomics, 4: 1686-1696.

Zhang, F., C. Li, H. Halfter and J. Liu, 2003. Delineating an oncostatin M-activated STAT3 signaling pathway that coordinates the expression of genes involved in cell cycle regulation and extracellular matrix deposition of MCF-7 cells. Oncogene, 22: 894-905.