Remodelling the cellular calcium homeostasis with a synthetic dihydropyrimidone, Nifetepimine: A targeted approach towards immune-rejuvenation and cancer regression

Parimal C. Sen, Ph.D.
Senior Professor
Division of Molecular Medicine
Dean of Studies
Bose Institute, Kolkata, India
Email: parimalsen.boseinst@gmail.com/parimal@jcbose.ac.in

National Institute of Biology, Ljubljana, Slovenia
September 18, 2014
Calcium: An Ubiquitous Cellular Signal

In the course of evolution, a number of agents have emerged as carriers of signals that are essential for the correct functioning of cell life. \( \text{Ca}^{2+} \) is the most versatile: other messengers are normally committed to the regulation of a single cell function, or at most a few of them, \( \text{Ca}^{2+} \) on the other hand regulates a plethora of cellular processes, beginning with the origin of new cell life, its growth, proliferation, differentiation and ending with its termination in the process of programmed cell death.

How is the calcium balance within the cell regulated???

- Calcium pumps
  - Plasma membrane \( \text{Ca}^{2+} \) ATPase (PMCA)
  - Sarco (endo) plasmic reticulum \( \text{Ca}^{2+} \) ATPase (SERCA)
  - Secretory pathway \( \text{Ca}^{2+} \) ATPase (SPCA)

- Voltage-gated channels
The SERCA pump

Calcium ions impact nearly every aspect of cellular life. It nearly triggers all cellular functions for example, contraction of myofilaments, secretion of hormones and neurotransmitters and modulation of metabolism, to cite a few. Moreover, Ca\(^{2+}\) also has a major function in triggering mitotic division in numerous cell types (e.g., T lymphocytes and of oocytes) and, conversely, in the regulation of cell death.

The first ATP-driven Ca\(^{2+}\) transport system, which was later identified as the SERCA pump, was discovered in a skeletal muscle fraction. SERCA actively pumps two calcium ions from the cytosol to the lumen of the ER. The pump exhibits 3 major isoforms; SERCA1a,b; SERCA2a-2c and SERCA 3a-3f.
Calcium signaling plays a significant role in T lymphocyte survival and activation. Lymphocyte activation produces modifications of the calcium storage and release characteristics of the cell [Clementi E et al (1994). *Eur.J.Immunol*. 24, 1365-1371] and down modulation of the expression of some specific SERCA isoenzymes is involved in the control of cell proliferation and of lymphocyte activation.

Thus the SERCA pump plays a critical role in the process of lymphocyte activation.
Cancer acts as a most devastating disease, because in addition to the disease itself, tumor progression is also associated with immunosuppressive conditions within the host. Indeed, malignant cells often use a variety of mechanisms to evade destruction offered by the immune system; by damaging the immune effector cells during the course of disease progression [Gastman BR et al (2000) Blood, 95,2015-2023].

The calcium homeostasis is often jeopardised in T lymphocytes during cancer development which ultimately causes apoptosis in T cells. In fact cancer cells liberate several soluble immunosuppresant like Prostaglandin E2 which have been found to alter T cell function...
Cancer acts as a most devastating disease, because in addition to the disease itself, tumor progression is also associated with immunosuppressive conditions within the host. Indeed, malignant cells often use a variety of mechanisms to evade destruction offered by the immune system; by damaging the immune effector cells during the course of disease progression [Gastman BR et al (2000) Blood, 95, 2015-2023].

Calcium homeostasis is often jeopardised in T lymphocytes during cancer development which ultimately causes apoptosis in T cells. In fact cancer cells liberate several soluble immunosuppresant like Prostaglandin E2 which have been found to alter T cell function.

Thus any compound/drug that can reorganise the cellular calcium homeostasis might be effective to recover T cells from this immunosupression.
CD4⁺T cells exhibited a significant percent of death on incubation with the MCF-7 supernatant which was also associated with an up-regulation in SERCA3 expression. To confirm the contribution of SERCA3 in tumor-induced CD4⁺ T cell apoptosis, firstly, SERCA3 gene was over-expressed in CD4⁺ T cell. The SERCA3 over-expressed CD4⁺ T cells furnished significant apoptosis (29.4%) even in the absence of the MCF-7 supernatant. In our second approach, transient silencing of SERCA3 gene protected the CD4⁺ T cells from the tumor supernatant-induced apoptosis where we observed that SERCA3 knocked-out cells underwent significantly lesser apoptosis (17.5%) even upon incubation with the MCF-7 supernatant. Thus our findings clearly suggest the involvement of SERCA3 during CD4⁺ T cell apoptosis under tumor induced condition.
As a profound reorganization of calcium homeostasis by down modulating SERCA expression may be of great help for both lymphocyte activation as well as cancer regression, we decided to study the consequences of SERCA pump down regulation with some of its known inhibitors. Several inhibitors of the SERCA pump are commercially available. Among the potent inhibitors are Thapsigargin, Nifedipine, Vanadate, Verapamil, Lanthanum Chloride and others.

However a major drawback with all these commercially available inhibitors are, concentrations at which they produce effective inhibition of the SERCA pump, lead to induce toxicity in normal cells of the body.

Thus we have searched for an effective SERCA pump inhibitor that can produce substantial inhibition without causing any major damage to normal body cells.
A synthetic SERCA pump modulator: Nifetepimine (NFTP)

Our quest led us to the synthesis of Nifetepimine [ethyl-4-(3-nitro)-phenyl-6-methyl-2-oxo-1,2,3,4 tetrahydropyrimidine-5 carboxylate], a synthetic analog of nifedipine, which down regulates SERCA pump activity without producing any significant damage towards normal cells.

Results clearly highlight that NFTP down regulates SERCA activity prominently at concentrations which produces no damage to normal cells.
SERCA3 over-expression causes ER stress-induced apoptosis

We investigated how upregulation in SERCA3 expression induced by the tumor supernatant produced death in CD4+ T cells. We observed that SERCA3 over-expression in CD4+ T cells was associated with the induction of ER stress that results in caspase activation and ultimately cell death. Conditions of both calcium overload and calcium depletion from the ER results in development of ER stress. Our findings clearly illustrated that treatment of CD4+ T cells with the tumor supernatant resulted in the activation of caspase 9 and caspase 3 leading to cell death.
CD4+ T cell restoration by nifetepimine involves down regulation of SERCA3 expression.

Nifetepimine down-regulated SERCA3 expression in a time-dependent fashion both at mRNA and protein levels while SERCA2b expression remained unaltered.

Our investigation revealed that SERCA3 expression that was prominently up-regulated in CD4+ T cells treated with cell-free MCF-7 and primary tumor supernatants, was thwarted by nifetepimine thereby ensuing in down-regulation of SERCA3 expression to relieve CD4+ T cells from tumor-induced apoptosis.
CD4+ T cell restoration by nifetepimine involves down regulation of SERCA3 expression.

Nifetepimine down-regulated SERCA3 expression in a time-dependent fashion both at mRNA and protein levels while SERCA2b expression remained unaltered.

Our investigation revealed that SERCA3 expression that was prominently up-regulated in CD4+ T cells treated with cell-free MCF-7 and primary tumor supernatants, was thwarted by nifetepimine thereby ensuing in down-regulation of SERCA3 expression to relieve CD4+ T cells from tumor-induced apoptosis.

These observations elucidate that the changes in the SERCA3 expression patterns are important for nifetepimine-mediated protection of CD4+ T cells from tumor-induced apoptosis.
Tumor shed PGE$_2$ is responsible for CD4$^+$ T cell apoptosis

Prostaglandin E2 is a major soluble immunosuppressant produced by the tumor, so we checked for the involvement of PGE$_2$ in mediating the process of T cell death.

We thereby transfected the tumor cells with the Cox-2 siRNA which blocked the release of PGE$_2$ in cell free tumor supernatant. Interestingly when the CD4$^+$ T cells were treated with this supernatant, the percentage of CD4$^+$ T cell apoptosis declined prominently. The enhancement in the SERCA3 expression level was also not observed on treating the CD4$^+$ T cell with the supernatant obtained from the Cox-2 siRNA transfected cells.
Tumor shed PGE$_2$ is responsible for CD4$^+$ T cell apoptosis

Prostaglandin E2 is a major soluble immunosuppressant produced by the tumor, so we checked for the involvement of PGE$_2$ in mediating the process of T cell death.

We thereby transfected the tumor cells with the Cox-2 siRNA which blocked the release of PGE$_2$ in cell-free tumor supernatant. Interestingly when the CD4$^+$ T cells were treated with this supernatant, the percentage of CD4$^+$ T cell apoptosis declined prominently. The enhancement in the SERCA3 expression level was also not observed on treating the CD4$^+$ T cell with the supernatant obtained from the Cox-2 siRNA transfected cells.

These findings therefore conclude that PGE$_2$ released in the tumor supernatant is primarily responsible for CD4$^+$ T cell killing and over-expression of SERCA3.
Our findings indicate that nifetepimine down-modulate SERCA3 expression to protect cells from tumor-induced apoptosis. Nifetepimine blocks caspase activation produced by the MCF-7 supernatant and thereby ameliorates the CD4+ T cell from tumor-induced immune-suppression. The apoptosis produced by the tumor supernatant was aborogated in the presence of caspase 3 (z-DEVD-FMK), caspase 9(z-LEHD-FMK), and pan-caspase inhibitor (z-VAD-FMK) thereby justifying the involvement of the caspases in this apoptotic cascade.
Nifetepimine-induced SERCA3 down modulation causes membrane translocation of PKC isoforms.

Calcium being a ubiquitous second messenger, interacts with the growth promoting protein kinase C (PKC) resulting in activation of the downstream signaling cascade. It is acknowledged that the ‘conventional’ PKC isoforms, PKC α and the ‘novel’ isoform PKC θ are the major isoforms of the PKC family involved in T cell activation, with PKC α lying upstream of PKC θ in the activation process.

Membrane translocation of PKCα and PKCΘ are favored by Nifetepimine

It is observed that the tumor burden significantly decreased the membrane expression levels of both the PKC isoforms. However, treatment with nifetepimine substantially increased the expression levels of PKC α and PKC θ in the membrane, thereby protecting the CD4+ cells from tumor-induced apoptosis.
Genetic manipulation of SERCA3 alters the membrane translocation of the PKC isoforms

Interestingly, it was observed that in the SERCA 3 over expressed cells membrane translocation of both the PKC isoforms was significantly reduced. Even on treating the transfected cells with nifetepimine, membrane translocation of the PKC isoforms was unaffected.

Contrastingly, when we checked the PKC isoforms expression status in the SERCA 3 siRNA transfected cells it was found that membrane expression levels of both the PKC isoforms was enhanced even in the absence of nifetepimine.

These findings strongly suggest that SERCA 3 down regulation by nifetepimine not only blocks apoptosis but also favors survival of the T cells from tumor-induced apoptosis.
Nifetepimine-mediated SERCA3 down modulation promotes NF-κB activation.

Nuclear Factor-kappa B is an important transcription factor that regulates T cell homeostasis and activation. Hence, we next evaluated the role of this transcription factor in nifetepimine-mediated CD4+ T cell restoration against tumor-induced apoptosis.

Our results depict that while MCF-7 supernatant retarded nuclear translocation of p65NF-κB in CD4+ T cells, nifetepimine efficiently stimulated NF-κB activity in these cells even in the presence of the tumor supernatant. It is known that the degradation of p50IκBα that retain NF-κB in the cytosol, and subsequent release of p65NF-κB requires prior phosphorylation of IκBα at Ser32 and Ser36 residues. Supporting our above-mentioned results, phosphorylated IκBα level was elevated in cytosol of CD4+ T cells upon nifetepimine treatment even in the presence of tumor supernatant.
Nuclear translocation of NF-κB was altered in SERCA3 over expressed and SERCA3 knockdown cells.

We observed that the nuclear translocation of p65 was substantially decreased in SERCA3 over expressed cells. The phosphorylation status of IκBα was low in the SERCA3 over expressed cells and the nuclear translocation of NF-κB was thereby decreased.

On the other hand in the SERCA3 knocked down cells, pIκBα levels were high in the cytosol and thus the nuclear translocation of p65 was favored even on treatment with the MCF-7 supernatant in the absence of nifetepimine.
Validation of the immunoprotective role of Nifetepimine in tumor bearing mice models

Swiss albino mice were intraperitoneally injected with 1x10⁶ EAC. After 1 week, different doses of Nifetepimine were administered intraperitoneally. Control mice received DMSO as carrier vehicle. Normal mice contained 15.92x10⁶ spleenic MNCs.

<table>
<thead>
<tr>
<th>Doses of Nifetepimine (µg/g body weight)</th>
<th>Spleenic MNC number (x10⁶) 3rd week</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.32</td>
</tr>
<tr>
<td>5</td>
<td>13.08</td>
</tr>
<tr>
<td>10</td>
<td>14.76</td>
</tr>
<tr>
<td>15</td>
<td>12.44</td>
</tr>
</tbody>
</table>

Tumor burden itself caused immunosupression as was evident from the depletion in spleenic MNC numbers. Nifetepimine at doses around 10µg/g body weight restored the depressed cell number to normal level.
Involvement of SERCA in the Nifetepimine-mediated immunorecovery in tumor-bearing mice models

FACS analysis reveals that tumor burden significantly decreased CD4+ T cell number, whereas treatment with nifetepimine significantly restored the depleted cell number. These CD4+ T cell populations were further gated to determine the changes in the SERCA3 expression level. Interestingly, it was observed that the rise in the SERCA3 expression levels in the tumor bearing mice was prominently reduced in the nifetepimine-treated sets which was also associated with rise in cytosolic [Ca2+] and NF-κB activation.
Involvement of SERCA in the Nifetepimine-mediated immunorecovery in tumor-bearing mice models

FACS analysis reveals that tumor burden significantly increased CD4+ T cell counts, whereas treatment with nifetepimine significantly restored the depleted cell number. These CD4+ T cell populations were further gated to determine the changes in the SERCA3 expression level. Interestingly, it was observed that the rise in the SERCA3 expression levels in the tumor bearing mice was prominently reduced in the nifetepimine-treated sets which was also associated with rise in cytosolic [Ca2+] and NF-κB activation.

These findings, therefore, hold promise that nifetepimine may have the potential to act an immune-restoring agent in cancer bearers.
Cancer cell

Nifetepimine

CD4+ T cell

Apoptosis

Cell Survival

PGE2

Caspase 3

Caspase 9

ER stress

NF-κB

Survival gene transcription

Cytoplasm

Nucleus

Sp1

SERCA3 promoter

PKC activation
Nifetepimine, a Dihydropyrimidone, Ensures CD4+ T Cell Survival in a Tumor Microenvironment by Maneuvering Sarco(endo)plasmic Reticulum Ca2+ ATPase (SERCA)∗

Received for publication, February 29, 2012, and in revised form, July 17, 2012. Published, JBC Papers in Press, July 31, 2012, DOI 10.1074/jbc.M112.357889

Swatilekha Ghosh†, Arghya Adhikary†, Samik Chakraborty†, Pinki Nandi†, Suchismita Mohanty†, Supriya Chakraborty†, Pushpak Bhattacharjee†, Sanhita Mukherjee†, Salil Pututanda†, Srabasti Chakraborty†, Arjit Chakraborty†, Gaurisankar Sa†, Tanya Das†, and Parimal C. Sen†

From the †Division of Molecular Medicine, Bose Institute, P1/12 Calcutta Improvement Trust Scheme VIIM Kolkata 700054, India, the †Department of Chemistry, Maulana Azad College, Kolkata 700013, India, and the †Department of Chemistry, Behala College, Kolkata 700060, India

Background: Tumor-induced SERCA3 up-regulation is a major cause of death of CD4+ T lymphocytes leading to immune suppression in cancer bearers.

Results: Nifetepimine down-modulates SERCA3 expression and thereby protects the lymphocytes from tumor-induced apoptosis.

Conclusion: The present finding strongly suggests nifetepimine as a potent immuno-restoring agent that protects T lymphocytes from tumor insult.

Significance: The results suggest that nifetepimine may be developed into a potent immuno-restoring agent in tumor-bearers.
A relationship between cancer and calcium has been repeatedly documented: it is acquiring special interest, as accumulating evidence now indicates that the altered cellular homeostasis of calcium may be involved in the abnormal cell proliferation that is a hallmark of the malignant transformation. The matter has aspects that are seemingly paradoxical: on one hand, the increase of cytosolic calcium, promotes cell proliferation. On the other hand, situations of calcium overload trigger apoptotic death pathways.
Nifetepimine induced apoptosis in human mammary carcinoma cells
Nifetepimine induced apoptosis in human mammary carcinoma cells

Nifetepimine is therefore found to induce death in MDAMB-231 and MDAMB-468 cells in a dose dependent manner producing about 45% cell death at dose of 50μM at 24 hours.
Our previous studies have already stated that nifetepimine as a modulator of cellular calcium homeostasis in T lymphocytes, we have evaluated the role of nifetepimine in regulating cytosolic calcium levels during induction of breast carcinoma cell apoptosis. Interestingly, our results clearly indicated that treatment of MDAMB-231 and MDAMB-468 cells with nifetepimine resulted in an increase in the cytosolic calcium concentration in a time dependent manner resulting in a situation of calcium overload and also activation of caspase 9 and caspase 3 resulting in cell death.
Swiss albino mice were intraperitoneally injected with $1 \times 10^6$ EAC. After 1 week, different doses of nifetepimine were administered intraperitoneally. Control mice received DMSO as carrier vehicle. After the 3rd week the viable EACs were counted from the peritoneal cavity of the mice.

<table>
<thead>
<tr>
<th>Doses of Nifetepimine (ug/g body wt)</th>
<th>EAC number*10^6</th>
<th>Spleenic MNC number*10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>442.27</td>
<td>11.32</td>
</tr>
<tr>
<td>5</td>
<td>133.23</td>
<td>13.08</td>
</tr>
<tr>
<td><strong>10</strong></td>
<td><strong>52.26</strong></td>
<td><strong>14.76</strong></td>
</tr>
<tr>
<td>15</td>
<td>16.55</td>
<td>12.44</td>
</tr>
</tbody>
</table>

*Control mice contained $15.96 \times 10^6$ splenic MNC.
Nifetepimine regresses cancer in tumor-bearing mice models

Swiss albino mice were intraperitoneally injected with $1 \times 10^6$ EAC. After 1 week, different doses of nifetepimine were administered intraperitoneally. Control mice received DMSO as carrier vehicle. After the 3rd week the viable EACs were counted from the peritoneal cavity of the mice.

<table>
<thead>
<tr>
<th>Doses of Nifetepimine (ug/g body wt)</th>
<th>EAC Viable (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.55</td>
</tr>
<tr>
<td>10 mg/kg body wt</td>
<td>13.08</td>
</tr>
<tr>
<td>20 mg/kg body wt</td>
<td>14.76</td>
</tr>
<tr>
<td>30 mg/kg body wt</td>
<td>12.44</td>
</tr>
</tbody>
</table>

It is observed that nifetepimine lessened the tumor burden considerably in a dose-dependent manner.
NIFETEPIMINE

Tumor Regression

Immune recovery

Cancer
Concluding Remarks

1. Nifetepimine down-regulates SERCA3 expression in CD4+T cells and thereby protects them tumor-induced apoptosis.

2. Nifetepimine by down-modulating the SERCA3 expression level regulates the intra-cellular calcium homeostasis and acts as a potent immunorestoring agent in tumor bearers.

3. Nifetepimine also effectively induces apoptosis in breast carcinoma cells by resulting in a situation of cytosolic calcium overload.

4. Thus cumulatively nifetepimine acts as a potent immune-rejuvenating and anti-carcinogenic agent thereby providing better hope for cancer patients worldwide.
OUR HOPE

Nifetepimine may emerge as a potential and effective immunorestoring and anti-cancer agent in the not so distant future.

“We have two options, medically and emotionally: give up or fight like hell.”
Acknowledgements

Prof. Parimal C. Sen
Prof. Tanya Das

Dr. Swatilekha Ghosh
Dr. Arghya Adhikary
Supriya Chakraborty
Salil Putatunda
Dr. Pinki Nandi
Dr. Arijit Chakraborty