REVIEW

Nelfinavir and other protease inhibitors in cancer: mechanisms involved in anticancer activity [version 2; peer review: 2 approved]

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Abstract

Objective: To review the mechanisms of anti-cancer activity of nelfinavir and other protease inhibitors (PIs) based on evidences reported in the published literature.

Methods: We extensively reviewed the literature concerning nelfinavir (NFV) as an off target anti-cancer drug and other PIs. A classification of PIs based on anti-cancer mode of action was proposed. Controversies regarding nelfinavir mode of action were also addressed.

Conclusions: The two main mechanisms involved in anti-cancer activity are endoplasmic reticulum stress-unfolded protein response pathway and Akt inhibition. However there are many other effects, partially dependent and independent of those mentioned, that may be useful in cancer treatment, including MMP-9 and MMP-2 inhibition, down-regulation of CDK-2, VEGF, FGF, NF-κB, STAT-3, HIF-1 alfa, IGF, EGFR, survivin, BCRP, androgen receptor, proteasome, fatty acid synthase (FAS), decrease in cellular ATP concentration and upregulation of TRAIL receptor DR5, Bax, increased radiosensitivity, and autophagy. The end result of all these effects is slower growth, decreased angiogenesis, decreased invasion and increased apoptosis, which means reduced proliferation and increased cancer cells death.

PIs may be classified according to their anticancer activity at clinically achievable doses, in AKT inhibitors, ER stressors and Akt inhibitors/ER stressors.

Beyond the phase I trials that have been recently completed, adequately powered and well-designed clinical trials are needed in the various cancer type settings, and specific trials where NFV is tested in association with other known anti-cancer pharmaceuticals should be sought, in order to find an appropriate place for NFV in cancer treatment.

The analysis of controversies on the molecular mechanisms of NFV hints to the possibility that NFV works in a different way in tumor cells and in hepatocytes and adipocytes.

Keywords

Nelfinavir, protease inhibitor, cancer, endoplasmic reticulum stress, unfolded protein response, Akt
In this article, we thoroughly review the literature published in this matter and analyze mainly the anti-cancer mechanisms of action of NFV.

Certain controversies regarding NFV activity in lipid metabolism will be considered in depth.

**Evidences of nelfinavir anti-cancer activity**

A partial response of Kaposi’s sarcoma patients to PIs was published in 1998 and good results with regression (six complete responses out of 10 patients)\(^1\). In 1999 Niehues et al.\(^2\) published complete regression of Kaposi’s sarcoma in a child treated with highly active anti-retroviral therapy (HAART). Sgardari et al. (2003) described also the inhibition of Kaposi’s sarcoma with protease inhibitors and they also mention that these drugs can antagonize vital properties of tumor cells like growth, invasion, tissue remodelling, angiogenesis and survival. They consider these effects to be a consequence of inhibition of invasion, matrix metalloprotease, proteasome and NF-κB signaling\(^3\). The possible mechanisms of PIs off target activity on tumor cells were described by pioneering work of Schmidtk et al. in 1999\(^4\): they observed that ritonavir was a modulator of proteasomal activity, allowed normal proliferation when used at low concentrations, but affected protein degradation when present at higher concentrations, and cell cycle was arrested.

Ikezoe et al.\(^5\) described that protease inhibitors increased cellular growth inhibition of all transretinoic acid (ATRA) on cell cultures of myelocytic leukaemia lines. Protease inhibitors also increased differentiation of acute myeloid leukemia cell lines.

In 2004, Ikezoe\(^6\) described the mechanisms involved in anti-cancer activity of protease inhibitors in myeloma cells.

The mechanisms involved in PIs anti-cancer activity are summarized in chronological order on Table 1.

**Nelfinavir and the ERS-UPR pathway**

NFV inhibits the proteases S1P and S2P that are involved in SREBP-1 maturation and other proteases necessary for protein maturation and folding (yet not fully identified) in the endothelial reticulum\(^7\).

Activation of the unfolded protein response (UPR) starts in the ER when abnormal accumulation of protein is detected\(^8\). This was investigated thoroughly in yeasts where detection of abnormal protein occurs through Ire1p/Ern1p-mediated signaling from the ER (in mammals there are three sensor proteins IRE1\(\alpha\), PERK and ATF6\(^9\)). UPR activation leads to the specific removal of 252 nucleotides intron from a precursor mRNA of the transcription factor HAC-1p, and the resulting mature mRNA HAC-1p is translated to produce active HAC-1p. This transcription factor translocates to the nucleus and promotes the transcription of chaperones like GRP78 that facilitates removal of abnormal proteins from the ER through retrotranslocation and final disposal by the ubiquitin-proteasome pathway\(^10\).

HAC1 precursor mRNA is constitutively expressed but not translated until Ire1p/Ern1p sensor removes the necessary nucleotides.
Thus the UPR is an intracellular signaling pathway where the ER “informs” the nucleus on the need to increase the levels of molecular chaperones and folding enzymes in order to maintain the ER homeostasis. Therefore UPR keeps unfolded proteins in the ER until they are correctly folded before they can go to their final destination. NFV seems to produce cellular stress by accumulation of misfolded or abnormal proteins in the ER, overwhelming the normal ER protein folding machinery. Chaperones bound to unfolded proteins in the ER initiate protein kinase cascades that inhibit translation, reverse translocation, activate ubiquitination enzymes, induce autophagia, and when stress is extreme, induce apoptosis.

Table 1. Mechanisms of action of Nelfinavir and other PIs in cancer.

<table>
<thead>
<tr>
<th>Author</th>
<th>Study performed in</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaedicke, 2002</td>
<td>Thymoma cells growing in syngeneic mouse</td>
<td>Ritonavir produces growth inhibition of tumors, apoptosis and affects proteosomal proteolysis. Non-transformed cell lines were relatively resistant to this activity. Accumulation of p21 (due to inhibition of proteolytic degradation).</td>
</tr>
<tr>
<td>Ikezoe, 2004</td>
<td>Human Multiple Myeloma cells</td>
<td>Growth arrest, apoptosis, blocked IL6 stimulated phosphorylation of STAT 3 and ERK ½. Decreased VEGF production.</td>
</tr>
<tr>
<td>Sgadari, 2002</td>
<td>Kaposi sarcoma cell lesions in nude mice</td>
<td>Anti-angiogenesis. Decrease of VEGF, bFGF. Decrease MMP2 activation.</td>
</tr>
<tr>
<td>Pajonk, 2002</td>
<td>PC-3 and DU-145 prostate cancer, U373 glioblastoma, and K562 and Jurkat leukemia cells</td>
<td>Saquinavir inhibited activation of NF-κB. Inhibited 20s and 26s proteasome activity. Sensitized the surviving cells to ionizing radiation. In a previous paper, this same group showed that proteasome is a direct target of radiation. This explains the synergism between proteasome inhibitors and ionizing radiation.</td>
</tr>
<tr>
<td>Olson, 2002</td>
<td>Cell culture of UMCC-1/VP cells which over-express MRP-1</td>
<td>Ritonavir inhibits the functional activity of the multidrug resistance-related-protein-1 (MRP-1). This characteristic was not shared by other PIs.</td>
</tr>
<tr>
<td>Zhou, 2004</td>
<td>Insulinoma cells</td>
<td>Nelfinavir decreased insulin stimulated phosphorylation of IRS-2 and Akt-Thr(308) in a dose-dependent manner. For 10 micromol/L of nelfinavir, the decrease in Akt phosphorylation was 55%.</td>
</tr>
<tr>
<td>Gupta, 2004</td>
<td>Human embryonic kidney cells</td>
<td>HIV PIs are breast cancer resistance protein inhibitors. This applies to ritonavir, saquinavir, and nelfinavir. Indinavir and amprenavir showed no inhibition on BCRP.</td>
</tr>
<tr>
<td>Piccinini, 2005</td>
<td>HL60 cells incubated with and without drug.</td>
<td>Saquinavir and nelfinavir inhibited proteasome activity at therapeutic dosages. Retroviral medication had no effect on proteasome.</td>
</tr>
<tr>
<td>Gupta, 2005</td>
<td>Tumor cell culture and xenografts.</td>
<td>Amprenavir, nelfinavir, and saquinavir inhibited Akt phosphorylation and exerted synergistic effects with radiotherapy.</td>
</tr>
<tr>
<td>Yang, 2005</td>
<td>Prostate Cancer Cells: LNCaP, DU145, PC3 and LNCaP xenografts in nude mice</td>
<td>NFV induces growth arrest and apoptosis of prostate cancer cells and blockade of androgen receptor, STAT3 and AKT. It also inhibits proliferation of LNCaP xenografts.</td>
</tr>
<tr>
<td>Yang, 2006</td>
<td>NSCLC cell culture and xenografts in nude mice.</td>
<td>NFV induces growth arrest, reduces Akt signalling, apoptosis and docetaxel sensitisation. It is responsible for up-regulation of p21, p27 and p53, down-regulation of Bcl-2 and MMP-2. NFV slowed proliferation and induced apoptosis in tumour xenografts mice without adverse systemic effects. Of the 3 PIs tested (saquinavir, ritonavir and NFV) NFV exerted the strongest inhibition on proliferation.</td>
</tr>
<tr>
<td>Chow, 2006</td>
<td>Liposarcoma and non liposarcoma cell lines</td>
<td>NFV induces apoptosis of liposarcoma cell through upregulation of SREBP-1. Authors consider that NFV is a new class of anti-liposarcoma agent.</td>
</tr>
<tr>
<td>Pore, 2006</td>
<td>Glioblastoma cells</td>
<td>NFV decreased VEGF expression and secretion under normoxia. NFV decreases VEGF through the PI3K/Akt pathway. NFV also decreased the hypoxic induction of VEGF and the hypoxic induction of HIF-1alpha. NFV’s effect was a decreased angiogenesis</td>
</tr>
<tr>
<td>Pore, 2006</td>
<td>in vivo Matrigel plug assay</td>
<td>NFV decreases VEGF expression through the transcription factor Sp1, which regulates VEGF promoter. It down-regulates HIF-1 alpha by decreasing translation.</td>
</tr>
<tr>
<td>Hampson, 2006</td>
<td>HPV transformed cervix carcinoma cells</td>
<td>Protease inhibitors inhibit S 26 proteasome blocking p53 degradation.</td>
</tr>
<tr>
<td>Ben-Romano, 2006</td>
<td>Cell culture of 3T3-L1 adipocytes</td>
<td>NFV induces oxidative stress that may lead to apoptosis (in adipocytes).</td>
</tr>
<tr>
<td>Gupta, 2007</td>
<td>Meningioma cells</td>
<td>Combination therapy with imatinib and NFV potentiated anti-proliferative activity of imatinib due to decrease in survivin and increase of Bax.</td>
</tr>
</tbody>
</table>
Study performed in cervical cancer line Neuroblastoma cells NFV improved vascular network.

Glioblastoma cell lines Cervical intraepithelial neoplasia 60 different cancer lines Authors describe a pathway NFV/ERS/CHOP/up regulation of trail receptor Inhibits proximal insulin receptor signalling which may explain insulin

Glioblastoma cells Results

Chemical systems biology Glioblastoma cells Melanoma cells NFV decreases Akt expression and enhanced radiosensitization in PTEN deficient glioblastoma cells.

Plastaras, 2008 NFV and Bortezomib (a proteosomal inhibitor) show synergy as apoptosis NFV decreased Akt expression and enhanced radiosensitization in PTEN

Lymphoblastoid B cells NFV and other ER stressor upregulate inhibin Beta E which shows anti-cancer resistant cell lines

NFV inhibits proteolysis of SREBP-1 by inhibiting site-2 protease (S2P). Inhibition of autophagy with hydroxychloroquine enhanced apoptotic effect of NFV.

NFV induced cell death in carboplatin-sensitive resistant ovarian cancer cell lines. NFV induced formation of ER-derived vacuoles and induced up-regulation of the hsp70 heat shock family member GRP78. It induced the unfolded protein response, which causes cell cycle arrest and apoptosis. Down-regulation of cell cycle regulatory proteins, especially cyclin D3.

NFV induced caspase dependent apoptosis and also caspase independent apoptosis via ER stress and autophagy.

NFV enhanced the effects of irradiation on endothelial cells.

Endoplasmic reticulum stress (ERS) response, as shown by increased expression of ERS markers, GRP78 and CHOP, and activation of ERS-associated caspase-4. Proteasome inhibition.

NFV upregulates TRAIL receptor DR5 which is an apoptosis inducing receptor.

NFV may act as a breast cancer resistance protein (BCRP) inhibitor with certain substrates.

NFV induced cell cycle arrest and apoptosis by down-regulation of cyclin D2 and surviving and suppressed transcriptional activation of NF-κB.

NFV improved vascular network.

Weak inhibition of multiple kinases is one of the causes of NFV anti-cancer activity, without severe side effects, but still having an impact on the system. Off targets of NFV are possibly: EGFR, IGF-1R, Akt2, Abl, FGFR, CDK2, ARK2, Fak1, PDK1, Ephrin receptors. The concept behind this research is that the whole is greater than the sum of the parts.

Authors describe a pathway NFV/ERS/CHOP/up regulation of trl receptor DR5. This pathway clearly interconnects NFV with apoptosis.

NFV and Bortezomib (a proteosomal inhibitor) show synergy as apoptosis inducers.

Growth retardation in vivo. Inhibition of PI3K-Akt-mTOR axis. Increased sensitivity to radiation.

Inhibition of S26 proteasome, impaired proliferation and increased apoptosis. Decreased phosphorylation of AKT, STAT3 and ERK ½. ER stress..

NFV may act as a breast cancer resistance protein (BCRP) inhibitor with certain substrates.

NFV inhibits proteolysis of SREBP-1 by inhibiting site-2 protease (S2P). Inhibition of autophagy with hydroxychloroquine enhanced apoptotic effect of nelfinavir. Accumulation of SREBP-1 produced ER stress. Decreases FAS.

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Mechanism of action of Nelfinavir in cancer

![Diagram of Nelfinavir's mechanisms of action in cancer](image)

**Figure 1.** Simplified mechanisms of action of nelfinavir in cancer. ERS: Endoplasmic reticulum stress.

**Figure 2.** A more detailed view of nelfinavir’s action on ER stress.
Figure 4. SREBP is synthesized as an ER transmembrane protein and transported to the Golgi upon appropriate stimulus. For activation of SREBP it is necessary that luminal S1P (a protease) cleaves first, followed by intramembrane S2P (another protease) to liberate the transcriptionally active amino-terminal segments of nSREBP. NFV inhibits S1P and S2P, so that transcriptionally active SREBP is not produced. Accumulation of inactive SREBP is one of the UPR initiators.
Table 2. Nelfinavir anti-cancer activity in different tumor tissues*.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Reference</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocarcinoma</td>
<td>Sun, 2014(^1)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Diffuse B cell lymphoma</td>
<td>Petrich, 2012(^2)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Kast, 2012(^3)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>Pan, 2012(^4)</td>
<td>Clinical Trial (Phase I)</td>
</tr>
<tr>
<td>HER 2 positive, breast cancer cells</td>
<td>Shim, 2012(^5)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>Kraus, 2014(^6)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>Kraus, 2013(^7)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Cancer stem cells expressing embryonic genes**</td>
<td>Darini, 2013(^8)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Kas, 2013(^9)</td>
<td>Clinical</td>
</tr>
<tr>
<td>Castration resistant prostate cancer</td>
<td>Mathur, 2014(^10)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Medullary thyroid cancer</td>
<td>Kushchayeva, Jensen, Recupero, 2014(^11)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Medullary thyroid cancer</td>
<td>Kushchayeva, Jensen, Burman, 2014(^12)</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Alonso-Basanta, 2014(^13)</td>
<td>Clinical Trial, Phase I</td>
</tr>
<tr>
<td>Rectal cancer</td>
<td>Buijsen J, 2013(^14)</td>
<td>Clinical Trial, Phase I</td>
</tr>
<tr>
<td>Refractory adenoid cystic carcinoma</td>
<td>Hoover, 2014(^15)</td>
<td>Clinical Trial, Phase II</td>
</tr>
</tbody>
</table>

*Saquinavir-NO has been tested in human melanoma cells with good results*\(^7\).

**It is necessary to underscore the finding that cancer stem cells expressing embryonic genes like Oct4, Sox2 and others, are particularly prone to apoptosis when PIs are used, particularly iopinavir (nelfinavir and saquinavir are also effective in this matter).

**Summary of Nelfinavir anticancer mechanisms**

- Upregulates p21, p27, p53
- Downregulates Bc12 and MMP2/9
- Endoplasmic Reticulum Stress
- Akt blocking
- Upregulation of SREBP-1 Precursors and downregulation of SREBP-1 activity
- Weak inhibition of multiple Kinases like EGFR, IGF1R
- Decrease of VEGF and HIF
- Inhibition of ERK1/2
- Inhibition of FAS (Fatty acid synthase)
- STAT 3 inhibition AR inhibition

**Figure 5. Summary of NFV anti-cancer mechanisms.**
Table 3. Summary of clinical trials performed with NFV as an anti-cancer drug.

<table>
<thead>
<tr>
<th>PROTOCOL</th>
<th>CHARACTERISTICS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT 0145106</td>
<td><strong>SOLID TUMORS:</strong> A phase I trial of nelfinavir (Viracept) in adults with solid tumors</td>
</tr>
<tr>
<td>NCT 00436735</td>
<td><strong>SOLID TUMORS:</strong> Nelfinavir in treating patients with metastatic, refractory, or recurrent solid tumors</td>
</tr>
<tr>
<td>NCT 02080416</td>
<td>Non-Hodgkin Lymphoma, Hodgkin Lymphoma, Kaposi Sarcoma, Gastric Cancer, Nasopharyngeal Cancer, EBV, Castleman Disease: nelfinavir for the treatment of gammaherpesvirus-related tumors</td>
</tr>
<tr>
<td>NCT 00589056</td>
<td><strong>NSCLC:</strong> NFV, radiation therapy, cisplatin, and etoposide in treating patients with stage III non-small cell lung cancer that cannot be removed by surgery</td>
</tr>
<tr>
<td>NCT 01068327</td>
<td><strong>Pancreatic cancer:</strong> stereotactic radiation therapy, nelfinavir mesylate, gemcitabine hydrochloride, leucovorin calcium, and fluorouracil in treating patients with locally advanced pancreatic cancer</td>
</tr>
<tr>
<td>NCT 01925378</td>
<td><strong>Cervical intraepithelial neoplasia:</strong> a phase II single-arm intervention trial of nelfinavir in patients with grade 2/3 or 3 cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>NCT 01959672</td>
<td><strong>Pancreatic cancer:</strong> nelfinavir mesylate in treating patients with locally advanced pancreatic cancer</td>
</tr>
<tr>
<td>NCT 01079286</td>
<td><strong>Renal cell cancer:</strong> study of nelfinavir and temsirolimus in patients with advanced cancers</td>
</tr>
<tr>
<td>NCT 00704600</td>
<td><strong>Rectal cancer:</strong> nelfinavir, a phase I/phase II rectal cancer study</td>
</tr>
<tr>
<td>NCT 01065844</td>
<td><strong>Adenoid cystic cancer:</strong> nelfinavir in recurrent adenoid cystic cancer of the head and neck</td>
</tr>
<tr>
<td>NCT 01164709</td>
<td><strong>Hematologic cancer:</strong> nelfinavir mesylate and bortezomib in treating patients with relapsed or progressive advanced hematologic cancer</td>
</tr>
<tr>
<td>NCT 01086332</td>
<td><strong>Pancreatic cancer:</strong> evaluation of nelfinavir and chemoradiation for pancreatic cancer</td>
</tr>
<tr>
<td>NCT 01485731</td>
<td><strong>Cervical cancer:</strong> safety study of nelfinavir + cisplatin + pelvic radiation therapy to treat cervical cancer</td>
</tr>
<tr>
<td>NCT 02024009</td>
<td><strong>Pancreatic cancer:</strong> systemic therapy and chemoradiation in advanced localised pancreatic cancer - 2</td>
</tr>
<tr>
<td>NCT 00915694</td>
<td><strong>Glioblastoma:</strong> nelfinavir, radiation therapy, and temozolomide in treating patients with glioblastoma multiforme</td>
</tr>
<tr>
<td>NCT 01108666</td>
<td><strong>NSCLC:</strong> proton beam radiation with concurrent chemotherapy and nelfinavir for inoperable stage III non small cell lung cancer (NSCLC)</td>
</tr>
<tr>
<td>NCT 00791336</td>
<td>Study to evaluate using nelfinavir with chemoradiation for NSCLC</td>
</tr>
<tr>
<td>NCT 02207439</td>
<td><strong>Larynx carcinoma:</strong> A phase II of nelfinavir, given with definitive, concurrent chemoradiotherapy (CTRT) in patients with locally-advanced, human papilloma virus (HPV) negative, squamous cell carcinoma larynx</td>
</tr>
<tr>
<td>NCT 01020292</td>
<td><strong>Glioma:</strong> nelfinavir and concurrent radiation and temozolomide in patients with WHO grade IV glioma</td>
</tr>
<tr>
<td>NCT 01555281</td>
<td><strong>Myeloma:</strong> nelfinavir and lenalidomide/dexamethasone in patients with progressive multiple myeloma that have failed lenalidomide-containing therapy</td>
</tr>
<tr>
<td>NCT 01728779</td>
<td><strong>Metastatic lesions of the lung, liver or bone:</strong> stereotactic body radiation with nelfinavir for oligometastases.</td>
</tr>
<tr>
<td>NCT 00233948</td>
<td><strong>Liposarcoma:</strong> nelfinavir mesylate in treating patients with recurrent, metastatic, or unresectable liposarcoma</td>
</tr>
<tr>
<td>NCT 02188537</td>
<td><strong>Myeloma:</strong> nelfinavir as bortezomib-sensitizing drug in patients with proteasome inhibitor-nonresponsive myeloma</td>
</tr>
<tr>
<td>EudraCT Number:</td>
<td><strong>Pancreatic cancer:</strong> a phase II study in patients with locally advanced pancreatic carcinoma: ARC-II – Akt-inhibition by nelfinavir plus chemoradiation with gemcitabine and cisplatin</td>
</tr>
<tr>
<td>2008-006302-42</td>
<td>University of Oxford</td>
</tr>
</tbody>
</table>
Interactions of NFV and PIs with other drugs

Indinavir and NFV increase anti-malarial action of artemisinin in vitro on Plasmodium falciparum, but artemisinin has also a off target anti-cancer activity. Thus it is reasonable to raise the question: may the association of artemisinin with NFV increase anti-cancer activity?

Another research team has included both, NFV and artemisinin, in a multidrug repurposed protocol (CUSP 9) for the treatment of relapsed glioblastoma.

Celecoxib is an ER stressor that may enhance NFV anti-tumor activity.

Chloroquine and hydroxycloroquine are autophagy inhibitors and may work synergistically with NFV, downregulating autophagy and increasing apoptosis.

Nelfinavir may produce overproduction of mcl1 through upregulation of Erk ½, which would reduce apoptosis. The problem can be solved adding sorafenib.

In breast cancer cells, tamoxifen enhances anti-cancer activity of NFV. This synergism was independent of the estrogen receptor status so that the authors consider that the association of NFV and tamoxifen may be advantageous even in patients with no hormone responsive tumors.

Saquinavir has an interesting off target effect: it decreases intracellular ATP in adipocytes. If this effect is similar in tumor cells, an association with metformin and 2-deoxyglucose may produce anti-cancer activity. There is growing interest on metabolic perturbations in cancer therapy and saquinavir may play a role in this field.

Clinical trials

A phase I dose escalation trial performed in 2014 established a MTD (maximum tolerated dose) of 3125 mg twice daily and described that 45% of patients with solid tumors treated with this dose decreased AKT activity and increased ERS indicators. This indicated a possible benefit in neuroendocrine tumor patients and also established that dose limiting toxicity consists in neutropenia.

The dose (3125 mg bid) is more than twice the dose used in HIV treatment. But lower doses, in the range of those used in HIV treatment have been tested, combining nelfinavir with chemoradiotherapy in pancreatic cancer with evidence of efficacy. No control group was used in this research, so comparison was established with known data from previous publications, mainly the favourable possibility of tumor resection after treatment.

Even lower doses (625 mg and 1250 mg bid) were tested in a phase I trial of NSCLC in stages IIIA/IIIB combined with chemoradiotherapy. In nine out of 12 patients a PET scan was available post-treatment with 100% overall response (56% complete response and 44% partial response). Unfortunately, in this trial there was no control group; 50% of the patients (six out of 12) lived for more than 22 months after treatment; 25% (three out of 12) lived without disease for more than 32 months. The results may be considered favourable, even without a control group.

Buijsen et al. recommend a dose of 750 mg NFV bid for a phase II trial of this drug in combination with chemoradiotherapy in locally advanced rectal cancer.

Ongoing phase II trials are mainly in myeloma (associated with bortezomib or lenalidomide), glioblastoma patients (associated with chemoradiation), pancreas (associated with gemcitabine and radiation) and lung. (See http://www.clinicaltrials.gov).

The first results of clinical trials did not show a meaningful improvement in the outcome of patients with refractory adenoid cystic carcinoma which is a malignant salivary gland tumor which usually has a poor prognosis. In this case NFV was used as monotherapy.

Why nelfinavir has a role to play in cancer therapy?

Akt activation is an important step in cancer phenotype and is a key player in acquisition and maintenance of cancer hallmarks. Akt is a nodal regulator of cellular survival pathways. There are no drugs at the present time that can inhibit this protein with a good safety profile. Wortmannin, perifosine and other chemicals designed for PI3K/Akt inhibition were too toxic for clinical use or have shown disappointing results, so they did not enter the medical practice. Insulin stimulation of Akt phosphorylation was reduced by 55% at achievable doses. At the same time there is clear evidence that it favours apoptosis and growth inhibition at clinically tolerable and achievable doses.

This anti-Akt activity of NFV can be reinforced by concomitant mTOR inhibition which results in synergistic cytotoxicity. This may be due to the fact that mTOR inhibition without Akt inhibition eliminates a negative biofeedback loop on Akt, producing increased phosphorylation of Akt. According to Sarbassov this negative feedback is born in the mTORC2 complex.

According to Carracedo, this negative feedback loop goes as far as PI3K (Figure 6).

mTOR inhibitors have become a new and important tool against cancer, for example in renal cell carcinoma. But the negative biofeedback loop on Akt must be solved to achieve really good results. NFV could be the clue.

But the most important anti-tumor activity of NVR is not limited to Akt inhibition but ER stress and UPR which may be one of the pathways leading to apoptosis.

Additional features of NFV and other protease inhibitors are

1) the ability to sensitize cancer cells to chemoradiotherapy,
2) anti-angiogenesis by decreasing VEGF/HIF expression,

Figure 6. Negative feedback loop.
Figure 7. Mechanism of synergy between NFV and mTOR inhibitors. At the left is depicted the pathway under normal or pathological circumstances; in the middle drawing mTOR inhibition is counterbalanced by Akt activation due to loss of the negative biofeedback circuit; on the right, inhibition of both, mTOR and Akt may result in increased anti-cancer results.

3) decreased expression of FAS (fatty acid synthase),
4) the combination of radiation and PIs is well tolerated\textsuperscript{39,94},
5) NFV cancer cell killing ability can easily be enhanced with other ER stressors like celecoxib\textsuperscript{79}. Cho \textit{et al.} found enhanced killing of chemoresistant breast cancer cells after celecoxib treatment that aggravated ER stress; perillyl alcohol is another stress aggregator that has been used with that purpose\textsuperscript{96},
6) In head and neck cancer related to HPV, NFV produced down-regulation of Akt and radiosensitization\textsuperscript{97},
7) NFV not only down-regulates Akt but also MAPK (in adenoid cystic cancer)\textsuperscript{98}, and retards oral cell proliferation including normal keratinocytes and squamous cell cancer\textsuperscript{99},
8) There are evidences, at least in pancreatic cancer, that NFV dependent down-regulation of Akt is independent of the mutational status of K-ras\textsuperscript{100},
9) There is clear evidence (in glioblastoma) of the relation between NFV and apoptosis through the following pathway\textsuperscript{46}:

10) Down-regulation of MMP-9 (reduced expression and secretion of MMP-9 by human preadipocytes)\textsuperscript{64,101},
11) Increased apoptosis by NFV when associated with anti-autophagy drugs like chloroquine or hydroxychloroquines, particularly in triple negative breast cancer cells\textsuperscript{102}.

Possible controversies
The SREBP pathway for regulation of fat metabolism is initiated through proteolytic cleavage of precursor forms of the SREBPs (125 Kd protein) in ER membranes. When cells are in need of sterol, the precursor SREBPs are hydrolyzed by a 2-step mechanism involving membrane-bound serine protease S1P and a metalloprotease S2P. The N-terminal fragment of SREBP (nSREBP) is a 68 Kd protein that translocates to the nucleus where it works as a promoter-enhancer, binding to sterol regulatory elements located in DNA and activates gene transcription (Figure 4). The nuclear SREBP can be rapidly degraded by a proteasome-mediated mechanism. This provides regulation of gene transcriptional activities\textsuperscript{103}.

Transgenic mice over-expressing the constitutively active nuclear forms of the SREBPs (nSREBPs) revealed that overexpression of SREBP-1 or SREBP-2 leads to activation of genes involved in the cholesterol and fatty acid biosynthesis cascades. These transgenic mice displayed the classical features of generalized lipodystrophy, similar to those found in patients under PI therapy\textsuperscript{104}.

Riddle \textit{et al.} in 2001\textsuperscript{105} found that PI therapy (they used ritonavir) induced the accumulation of activated SREBP-1 and SREBP-2 in the nucleus of liver and adipose tissues. As a consequence, fatty acid and cholesterol biosynthesis were increased in these tissues. The authors consider that lipodystrophy, hyperlipidemia, and insulin resistance, are the consequence of activated SREBP-1 and SREBP-2 accumulation in the nucleus of liver and adipose tissues. The possible mechanism for these events, according to their criteria is PI suppression of activated SREBP degradation in the nucleus. In summary, Riddles’s study showed that ritonavir induced lipid metabolism abnormalities through stabilization of activated SREBP-1 and SREBP-2 in the nucleus of liver and adipose tissues.

These findings are in contrast with those of Guan\textsuperscript{95,106} where NFV inhibited the nuclear translocation of the sterol regulatory element binding protein-1 (SREBP-1) in castration resistant prostate cancer and liposarcoma through inhibition of S1P. This led to accumulation of unprocessed SREBP-1.
Riddle et al. described accumulation of processed SREBP-1 in the liver and adipose tissue while Guan found accumulation of unprocessed SREBP1 in ER and Golgi with no translocation to nucleus in liposarcoma and castration resistant prostate cancer tissue.

The controversy may be explained in the following way:

1) There are three different isoforms of SREBP: SREBP-1a, SREBP-1c and SREBP-2.
2) SREBP-1a and -1c have different expression profiles: SREBP-1a is highly expressed in proliferating cells, such as cancer cells, while SREBP-1c is the predominant form in normal cells, particularly hepatocytes\textsuperscript{104}.
3) The target genes for the three SREBP isoforms are different.
4) Riddle et al. found increased SREBP-1 and two in the nucleus of liver and adipose tissues; these SREBPs are the active form (they make no difference between SREBP-1a and SREBP-1c).
5) Guan et al. found increased SREBP in Golgi in the inactive form (precursor) of tumor tissues treated with NFV.
6) It is possible that tumor tissues that overexpress SREBP-1a behave in a different way than liver and adipose tissue that overexpress SREBP-1c.
7) Riddle et al. tested ritonavir and Guam et al. tested NFV, so the pharmacological effects between these PIs may differ.

A second controversy that stems from the one described above is on the effect of NFV on FAS:

1) According to Guan et al.\textsuperscript{106}, NFV decreases expression of FAS in liposarcoma cells and castrate resistant prostate cancer as was depicted in Figure 3.
2) According to Lenhard et al. 2000\textsuperscript{105}, NFV increases expression of FAS in HepG2 cells (which show many of the normal biochemical functions of non tumor liver parenchymal cells).

May this difference be due to tissue-specific effects of NFV? Does NFV have different effects in tumor tissues and normal tissues?

To definitely solve these controversies, it is necessary to proceed with further experimental research, but the findings described above necessarily raise the doubt that mechanisms that work in tumor cells might be slightly different from those working in hepatocytes and adipocytes.

Possible negative aspects of PIs in cancer

Despite the anti-cancer activity of NFV and PIs, these drugs do not reduce the risk of developing cancer in HIV population\textsuperscript{108} and also exert certain depression of immunological functions, interfering with the differentiation program of monocytes into dendritic cells\textsuperscript{109}.

PIs increase the expression of P-glycoprotein (ABCB1) in Kaposi’s sarcoma cell lines increasing the multidrug resistance phenotype\textsuperscript{110}.

At the same time ABCB1 expression depends on Akt activation\textsuperscript{111} and NFV inhibits partially Akt. The final result of the two antagonistic aspects requires further research.

There are well known undesirable side effects with HIV PIs, like hyperlipidemia, insulin resistance and lypodystrophy (peripheral fat wasting and excessive central fat deposition). One of the main responsible mechanisms of these side effects is the suppression of the breakdown of SREBP in the liver and adipose tissues resulting in increased fatty acid and cholesterol biosynthesis. SREBP accumulation in adipose tissue causes lipodystrophy.

PIs suppress proteasome-mediated breakdown of nascent apolipoprotein (apo) B, resulting in the overproduction of triglyceride. Finally, PIs also suppress the inhibition of the glucose transporter GLUT-4 activity in adipose tissue and muscle. This contributes directly to insulin resistance and diabetes\textsuperscript{112}.

Hepatomegaly and hepatic steatosis are direct consequences of the metabolic alterations explained above\textsuperscript{106}.

New PIs with anti-cancer activity

In 2010 You et al.\textsuperscript{113} synthesized a new indinavir analogue with remarkable anti-cancer activity, similar to NFV: CH05-10. This drug achieved similar cytotoxicity to NFV but at lower concentrations, against leukaemia, melanoma, ovarian and prostate cancer cell lines.

In 2009 Saquinavir-NO was introduced\textsuperscript{114}; it showed interesting anti-cancer properties in melanoma xenografts with significantly lower toxicity than saquinavir.

Conclusions

The most relevant mechanisms of PIs anti-cancer activity are Akt inhibition and ER stress.

Following our exhaustive analysis of the current medical literature we conclude that NFV anti-cancer activity is mainly dependent on ER stress-UPR.

Akt inhibition plays also a very important role but is not the unique or main source of anti-cancer effects.

The evidences that support these conclusions are:

1) Even at very high doses of NFV (3,125 mg bid), Akt achieved a level of inhibition around 55% in cell culture\textsuperscript{20}.
2) NFV is at the same time a strong ER stressor and an Akt inhibitor.
3) Anti-cancer activity can be achieved at much lower doses than those necessary for Akt inhibition.
4) Increasing ER stress by adding Celecoxib to NFV enhances cytotoxicity.
5) Autophagy, which is one of the mechanisms cells use to survive increasing ER stress\textsuperscript{115}, is inhibited by adding chloroquine or hydroxychloroquine to NFV. In this case, apoptosis is significantly enhanced\textsuperscript{20, 102}.
6) The PIs with anti-cancer activity like NFV, ritonavir, saquinavir, and the experimental drug CH05-10 are strong ER stressors. Amprenavir is a PI that induces no ER stress and its anti-cancer activity is significantly weaker than that of NFV, although it has Akt inhibiting effects.

7) Ritonavir, which is ER stressor, shows anti-cancer activity although it does not down-regulate Akt at concentrations usually found in HIV patients.

8) Inhibition of proteasome with bortezomib has a synergistic effect with NFV apoptotic activity.

9) PIs can be classified regarding anti-cancer activity at clinically achievable concentration in patients in:

A) Akt inhibitors only: e.g. amprenavir
B) ER stressors only: e.g. ritonavir
C) Akt inhibitors and ER stressors: examples NFV and experimental PI CH05-10. Of course this is the group that shows stronger anti-cancer activity.

There is enough evidence of NFV anti-cancer effects and there is adequate knowledge of how this activity works, so that NFV deserves well designed phase II clinical trials, as adjunct cancer therapy.

Associations with proteasomal inhibitors, celecoxib and other cell stressor should also be investigated in the clinical setting due to possible synergy. Tamoxifen with NFV may show interesting results in breast cancer.

Although a large amount of publications, including reviews, have been written on NFV and other PIs in cancer, none has been dedicated to a thorough examination and analysis of the mode of action of these pharmaceuticals as off target drugs (with the exception of the review by Gantt et al.). It is hoped that this review will encourage an increment adequately powered and well-designed clinical trials in the various cancer types, beyond the phase I trials that have been recently performed, and specifically trials where these compounds may be tested in association with other known anti-cancer pharmaceuticals like NFV associated to bortezomib and hydroxychloroquine in myeloma, or mTOR inhibitors with NFV in HNSCC and many other possible combinations where the dual feature of NFV, ER stressor and Akt inhibitor, are required.

In myeloma NFV increases proteasome inhibition by bortezomib and may overcome resistance to proteasomal inhibitors. This is an action exclusive of NFV and not shared with other PIs, with the additional advantage that NFV shows the highest cytotoxic activity against primary myeloma cells.

If apoptosis is described as a cascade, then apoptosis stimulator drugs like NFV should be viewed as enhancers of this cascade. An initiator of the cascade is still necessary, for example chemoradiotherapy. After this initial step, apoptosis stimulator drugs increase the amount of cells entering this pathway. This might be one of possible reasons why nelfinavir alone has shown poor results in a clinical trial used as monotherapy.

This does not mean that NFV cannot act as an initiator, but the evidences show that it is prone to be an enhancer of apoptosis rather than an initiator.

Future directions
All the evidences presented in this review reinforce the concept that NFV is a useful drug in cancer treatment. It should be considered in association with chemoradiotherapy in the design of new protocols for diseases like multiple myeloma (in association with bortezomib and hydroxychloroquine) and prostate, pancreas and lung cancer where clinical trials are ongoing. New PIs are being developed with better anti-cancer profile like CH05-10 and saquinavir-NO and further development of new PIs with stronger anti-cancer activity, will probably go on in the future.

Competing interests
No competing interests were disclosed.

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This is a highly informative and comprehensive review on our current knowledge about the anti-cancer mechanism of nelfinavir. It also deals with the pros and cons of a possible repurposing of nelfinavir (and other HIV PIs) for cancer treatment.

The following amendments and corrections are suggested:

Proteasome: In the abstract, the proteasome is listed among the factors to be “down-regulated” by nelfinavir. Is the proteasome “downregulated” or “inhibited” in its activity? This might be a slight semantic inaccuracy but is important because it reflects different molecular mechanisms. The same applies to Akt, which, according to the author, is mentioned to be “inhibited” in some phrases and to be “downregulated” (as a protein?) by nelfinavir in other phrases.

Independent of this, there is also a controversy in the literature about the ability of nelfinavir to inhibit proteasomal activity. Some authors observed inhibition of proteasomal enzyme activity by HIV PIs, whereas proteasomal target genes such as HIFα and cyclins have unexpectedly not been described to accumulate in the presence of PIs. Instead, as mentioned in this review, several of these proteasomal target proteins were found to be downregulated by nelfinavir treatment.

Using yeast terminology (HAC1) to introduce human ER stress mechanisms might confuse the reader.

Fig.1: How does ER stress lead to proteasome inhibition?

I have problems with the author’s proposed classification of PIs into ER stress inducers and Akt inhibitors (Abstract and Conclusion). Ritonavir is listed as an ER stressor only (conclusions “9”), but has been identified by several researchers to cause both ER stress and Akt inhibition. Furthermore, according to our experience, the ability of PIs to reduce Akt activity appears to be dependent on the cell line(s) used. Our group also observed enhanced Akt phosphorylation by nelfinavir in cell lines with low endogenous Akt activity, and Akt activation is also a known response to ER stress.
A combination of nelfinavir with mTOR inhibitors is suggested by the author. There are some independent recent reports indicating that nelfinavir also acts as an mTOR inhibitor.

The ability of nelfinavir to induce oxidative stress should be mentioned, because this may represent one of the reasons why nelfinavir may exert ER stress or other anti-cancer effects. (Fig. 2, for example, suggests undefined “protease inhibition” as the only mechanism of nelfinavir-induced ER stress).

Some typos in the text and in the figures should be corrected (hidroxychloroquine; phosphorilation; endothelial (=endoplasmic?) reticulum; Bc12 (=bcl2? Fig. 5)).

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Author Response 03 Jun 2015

**Tomas Koltai**, Centro de Diagnostico y Tratamiento de la Obra Social del Personal de la Alimentación, Talar de Pacheco, Buenos Aires, Argentina

Thank you for your deep and knowledgeable revision of this manuscript. I agree with you about the inaccuracies mentioned and there will be corrected in a third version of the article.

Of course, down-regulation and inhibition in the molecular world are two different things, so it is wise to make the modifications you suggested: proteasome is inhibited and not down-regulated by NFV. Akt, being a protein is down-regulated and not inhibited. These will be modified in version 3.

Yeast terminology for proteins playing a role in proteosomal stress may be confusing, so it will be changed for proteins found in mammalian proteosomal stress.

The classification of PIs according their main anticancer activity is based on clinically achievable concentration of the drug. It is quite possible (I found no experimental evidence so far) that at very high concentrations, PIs are both ER stressors and Akt down-regulators. But at doses used in the clinical setting the classification I propose may represent actual differences between the different PIs.

Oxidative stress will be mentioned in version 3. I agree it is an important cause of ER stress.

Amendments will be made to figure 5 along with the remarks you kindly made. The association of NFV and mTOR inhibitors showed interesting results in naturally occurring squamous cell tumors in dogs in our experience (unpublished data). This probably means some sort of synergy between the two. This synergy is mentioned by many authors. NFV acting on mTOR will be particularly highlighted.

Thank you for your interesting revision. All issues you mentioned will be considered in version 3 of this article.

**Competing Interests:** No competing interests
The article by Koltai reviews the extensive literature concerning Nelfinavir as an anti-cancer drug. Overall the study makes a good update of the evidences of nefilnavir anti-cancer activity and its role in controlling ERS-UPR pathway. It would be interesting to include in the discussion information about the different trials that are currently carry on with Nelfinavir in different tumors.

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.