IJC
International Journal of Cancer

HIV-protease inhibitors for the treatment of cancer: Repositioning HIV protease inhibitors while developing more potent NO-hybridized derivatives?

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Mini Review

The possible use of HIV protease inhibitors (HIV-PI) as new therapeutic option for the treatment of cancer primarily originated from their success in treating HIV-related Kaposi's sarcoma (KS). While these findings were initially attributed to immune reconstitution and better control of oncogenic viral infections, the number of reports on solid tumors, KS, lymphoma, fibrosarcoma, multiple myeloma and prostate cancer suggest other mechanisms for the anti-neoplastic activity of PIs. However, a major drawback for the possible adoption of HIV-PIs in the therapy of cancer relies on their relatively weak anticancer potency and important side effects. This has propelled several groups to generate derivatives of HIV-PIs for anticancer use, through modifications such as attachment of different moieties, ligands and transporters, including saquinavir-loaded folic acid conjugated nanoparticles and nitric oxide (NO) derivatives of HIV-PIs. In this article, we discuss the current preclinical and clinical evidences for the potential use of HIV-PIs, and of novel derivatives, such as saquinavir-NO in the treatment of cancer.

Following characterization of the three-dimensional structure of the HIV-1 protease,[1] several HIV protease inhibitors (HIV-PIs) have been developed.[1,2] The inhibitors of HIV protease are peptidomimetics containing an analogue of the peptide bond between phenylalanine and proline at positions 167 and 168 of the gag-pol polyprotein, target of the HIV aspartyl protease. The first in class HIV-PI was saquinavir, and up to now, there are 10 HIV-PIs approved by the FDA, that is, saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, fosamprenavir, lopinavir, atazanavir, tipranavir and darunavir.[1,2]

Several lines of evidence indicate that in addition to the antiretroviral properties, HIV-PIs possess pleiotropic pharmacological actions, including anticancer effects.[3] The possible use of HIV-PIs as a new therapeutic option for the treatment of cancer primarily originated from their success in treating HIV-related Kaposi's sarcoma (KS).[3] While these findings were initially attributed to immune reconstitution and better control of oncogenic viral infections, a number of reports on treating tumors, for example, KS, lymphoma, fibrosarcoma, multiple myeloma and prostate cancer, suggests other mechanisms for the antineoplastic activity of HIV-PIs.

Although HIV-PIs are not expected to cross-react with human proteases, preclinical data show that their anticancer effect may in part be attributed to inhibition of endopeptidases, such as metalloproteases and proteasomes. Indeed, aberrant proteasome-dependent proteolysis may lead to the accumulation of pro-apoptotic proteins in malignant cells and matrix metalloproteases (MMPs) are supposed to allow local expansion of cancer via disruption of normal tissue structure and by promoting invasion of blood vessels and lymphatics by metastatic cells.[4–9]

HIV-PIs may also protect against virus-associated cancers. Hampson *et al.* 2006,[10] reported that lopinavir, indinavir and ritonavir inhibit *in vitro* the HPV E6-mediated proteasomal degradation of mutant p53 in E6-transfected C33A cells with a stable increase in the levels of nuclear p53 as consequence. Ritonavir has been found to efficiently target NFkB and to inhibit tumor growth and infiltration of EBV-positive lymphoblastoid B cells.[11] Also, HIV-PIs hamper KS-associated herpesvirus and cytomegalovirus replication *in vitro*[12] and HHV-8 shedding in HIV patients under HIV PI-based Highly Active Anti-Retroviral Therapy (HAART).[13]

Currently used chemotherapeutic drugs are being tested in combination with HIV-PIs, both in preclinical and clinical studies in order to evaluate whether the combination of cancer chemotherapy and HAART may achieve better response rates than antineoplastic therapy alone. However, HIV-PIs

Key words: HIV protease inhibitors, saquinavir-NO, cancer therapeutics
DOI: 10.1002/ijc.30529
History: Received 14 June 2016; Accepted 16 Nov 2016; Online 21 Nov 2016
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generally show low potency as anticancer drugs, requiring concentrations >10 μM for cellular activity.[14]

The recognition of HIV-PIs as potential antitumor agents has intensified the effort to understand their mechanism of action in cancer and to develop more potent derivatives. In this article, we discuss the current preclinical and clinical evidences for the potential use of HIV-PIs, and of novel derivatives, such as saquinavir-nitric oxide (Saq-NO), in the treatment of cancer.

Preclinical Studies

Anticancer effects of HIV-PIs

Inhibitory effects on tumor cell growth, proliferation, invasion and angiogenesis indicate that HIV-PIs may have valuable therapeutic effects in both hematological and solid malignancies. A summary of the HIV-PIs anticancer effects is presented in Table 1. Gills *et al.* found that 3 out of 6 HIV-PIs (ritonavir, saquinavir and nelfinavir) inhibited the growth of over 60 cancer cell lines derived from 9 different tumor types.[15,16] Nelfinavir was the most effective in blocking growth factor receptor activation and downstream Akt signaling, thus triggering caspase-dependent apoptosis, Endoplasmic Reticulum (ER) stress (ERS) and autophagy. Nelfinavir also forced tumor growth and up regulated markers of ERS, autophagy and apoptosis. Ritonavir, saquinavir and nelfinavir in particular, inhibited proliferation of NSCLC cells and drug-resistant breast cancer cell lines in the NCI60 cell line panel. In this system, nelfinavir's mechanism of action included both caspase-dependent and caspase-independent death followed by induction of ERS and autophagy. Knowing that inhibition of autophagy increased nelfinavir-induced death, autophagy in this case seemed to have a protective role. The antitumor effect of nelfinavir on NSCLC was confirmed *in vivo* using a xenograft model.[15,16]

HIV-PIs also affect cancer stem cells (CSCs) detected in different high-grade tumor types with poor prognosis. These cells exhibit an embryonic phenotype characterized by the expression of Oct-4, Nanog and Sox2. The ability of HIV-PIs to specifically target CSCs derived from tumors with distinct origins opens the prospect of using HIV-PIs to treat patients with aggressive malignances. Lopinavir was found to be particularly efficacious, as it abolished self-renewal and provoked apoptosis of CSCs, thus inhibiting formation of CSC-induced allografts *in vivo*.[17]

Apart from a direct tumoricidal effect, HIV-PIs suppress growth of adenocarcinomas of lung, breast, colon and hepatic origin by blocking angiogenesis and MMP activity.[18] Indinavir and saquinavir also inhibit the appearance and regression of angioproliferative KS-like lesions in nude mice. At concentrations achieved in patients, HIV-PIs inhibited endothelial- and KS-cell invasion and of MMP-2 activity.[19] Similarly, saquinavir and ritonavir inhibit growth and invasion of cervical intraepithelial neoplasia by reducing cellular expression and activity of MMP-2 and −9.[19] Nelfinavir declined Vascular Endothelial Growth Factor (VEGF) secretion under normoxic conditions, most likely through the PI3K/Akt pathway. Hypoxic induction of VEGF and the HIF-1α, a known regulator of the VEGF promoter, was also diminished under nelfinavir treatment.[20,21]

Radiosensitization and chemosensitization properties of HIV-PIs

Studies have shown that HIV-PIs are useful radiosenzitizers, as amprenavir, nelfinavir and saquinavir increase the cytotoxic effect of radiation on tumor cells.[22,23] This effect was confirmed *in vivo*, likely due to their potential to inhibit Akt phosphorylation, since administration of amprenavir or nelfinavir down-regulated the phosphorylation of Akt in SQ20B and T24 xenografts.[22] Nelfinavir and other PI-3K/Akt inhibitors, are effective pancreatic cancer radiosensitizers regardless of K-ras mutation status.[24] Nelfinavir sensitized pituitary adenoma cells to ionizing radiation probably through decreased phospho-S6 and the PI-3K-Akt-mTOR pathway.[25] Nelfinavir decreased Akt phosphorylation and enhanced radiosensitization in PTEN deficient, U251MG and U87MG glioblastoma cells.[26] Radiosensitization was also assessed *in vivo* using a tumor regrowth delay assay in nude mice implanted with U87MG xenografts.[26] Nelfinavir also increased the sensitivity of U251MG cells to temozolomide. These results support the use of nelfinavir in combination with radiation and temozolomide in clinical trials for patients with glioblastomas.[26] At clinically attainable concentrations, saquinavir's activity was potentiated in association with imatinib in neuroblastoma cells.[27] Similarly, Gupta *et al.* proposed that nelfinavir not only potentiates imatinib efficacy on meningiomas, but also abrogates resistance to imatinib by decreasing survivin protein levels.[28] In an *in vivo* assay, this combined application was found to be more effective than imatinib alone. Ritonavir, saquinavir, nelfinavir and lopinavir have been shown to sensitize AML primary cells for proteasome inhibitor bortezomib/carfilzomib even in bortezomib/carfilzomib-resistant myeloma cells.[29] Ritonavir enhanced the antiproliferative and proapoptotic effects of docetaxel in the hormonally independent DU145 prostate cancer cells. Furthermore, combined treatment of docetaxel and ritonavir dramatically inhibited the growth of DU145 cells present as tumor xenografts in BNX nude mice compared with either drug alone.[30] Docetaxel induced expression of CYP3A4 in DU145 xenografts and ritonavir completely blocked this induction. Ritonavir also inhibited NFκB DNA binding activity in DU145 xenografts. Induction of ERS and suppression of the PI-3K/Akt survival pathway as a potent chemosensitization approach was evaluated in castration resistant prostate cancer cells.[30] When co-treated with nelfinavir, the doxorubicin (DOX)-resistant breast cancer cell line, MCF-7/Dox, which shows a DOX-induced cytotoxicity at 48 hr post-exposure (DOX IC50) 15–20 fold higher than the parental MCF-7 cells, showed a significant reduction in DOX IC50.[31] Multiple exposures to nelfinavir inhibited both P-gp expression and efflux function, thus elevating intracellular DOX

Table 1. Preclinical studies on the HIV PIs anticancer effects

HIV-PI	Cancer type/cell line(s)	Mechanism of Action	Reference
Nelfinavir	Prostate cancer/LNCap	↓ AR-induced STAT3 and AKT signaling	32
	Liposarcoma/SW872 and LiSa2	↑ Apoptosis, cell cycle arrest via ↑ SREBP1	33
	Multiple myeloma/U266, MM1S, RPMI8226, OPM2,LP1, 293T, ARH77, primary myeloma cells	↓ Mcl-1	34,35
		↑ PERK phosphorylation and CHOP expression; ↓ AKT, STAT3 and ERK1/2	
	Various cancer types/NCI panel	↑ ER stress, autophagy, apoptosis	16
	Glioblastoma/U251, LN229, T98G, U87	↑ ER stress	21,36
		↓ Proteasome activity	
		↓ VEGF/HIF1alpha	
	Breast cancer/HCC1143, HCC1395, HCC1937, HCC1954, HCC2218, MCF-7, BT474 and HCC38	↓ HSP90	37
		↓ HER2-induced AKT and ERK signaling	
	Head and neck cancer/SQ20B	↓ VEGF/HIF1alpha	21
	Lung cancer/A549	↓ VEGF/HIF1alpha	21
	Thyroid cancer/TT and MZ-CRC-1	↑ RET signaling	38
		↑ Apoptosis, autophagy	
		↑ HSP90	
		Mitochondrial oxidative stress	
	Cervical cancer/Hela, SiHa and CaSki	↑ Apoptosis, cell cycle arrest	39
		↑ Mitochondrial ROS ↓ SOD-2	
Saquinavir	Prostate cancer/LNCap, DU-145, PC3	↓ NFkB	23
		↓ 26s Proteasome	
		↑ Apoptosis	
	Multiple myeloma/U266, RPMI8226, ARH77	↓ Mcl-1	34
		↓ AKT, STAT3 and ERK1/2	
	Various cancer types/NCI panel	↑ ER stress, autophagy, apoptosis	16
	Ovarian cancer/A2780, CAOV3, SKOV3, OVCAR3, TOV21G, OVCAR4, OVCAR5, OVCAR8, OVCA429, OVCA432	↑ ER stress, autophagy and apoptosis	40
	Leukemia/Jurkat	↑ Telomerase activity	41
	Lung cancer/A549	↓ Angiogenesis	18
		↓ MMPs	
	Colon cancer/SW480	↓ Angiogenesis	18
		↓ MMPs	
	Breast cancer/MDA-MB-468	↓ Angiogenesis	18
		↓ MMPs	
	Liver cancer/SK-HEP-1	↓ Angiogenesis	18
		↓ MMPs	
	Kaposi sarcoma(primary cells	↓ Angiogenesis	19
		↓ MMPs	
	Cervical cancer/primary cells	↓ MMP-2, MMP-9	42
Ritonavir	Multiple myeloma/U266, RPMI8226, ARH77	↓ Mcl-1	43
		↓ AKT, STAT3 and ERK1/2	
		↓ GLUT4	
	Prostate cancer/DU145	↓ NFkB binding activity	44

Table 1. Preclinical studies on the HIV PIs anticancer effects (Continued)

HIV-PI	Cancer type/cell line(s)	Mechanism of Action	Reference
	Various cancer types/NCI panel	↑ ER stress, autophagy, apoptosis	16
	Breast cancer/MCF7, T47D, MDA-MB-436, MDA-MB-231	↓ AKT, ↓HSP90	45
		↑ Cell cycle arrest	
	Cervical cancer/primary cells	↓ MMP-2, MMP-9	42
Indinavir	Leukemia/Jurkat	↑ Telomerase activity	41
	Lung cancer/A549	↓ Angiogenesis	18
		↓ MMPs	
	Colon cancer/SW480	↓ Angiogenesis	18
		↓ MMPs	
	Breast cancer/MDA-MB-468	↓ Angiogenesis	18
		↓ MMPs	
	Kaposi sarcoma(primary cells	↓ Angiogenesis	19
		↓ MMPs	
Lopinavir	CSCs	↑ Apoptosis	17
Atazanavir	Glioblastoma/U251, LN229, T98G, U87	↑ ER stress	36
		↓ Proteasome activity	
Amprenavir	Glioblastoma/U251, U87	↓ VEGF/HIF1alpha	20

concentrations, suppressing phosphorylated Akt levels and increasing unfolded protein response (UPR) transducers and ERS induced death sensor expression. This was confirmed *in vivo* using mice carrying MCF-7/Dox tumor xenograft.

Clinical Trials

Nelfinavir

The marked anticancer activity of HIV-Pis in the preclinical setting has propelled clinical investigations of these drugs in cancer patients. Table 2 shows the trials of HIV-PIs listed in http://www.clinicaltrials.gov. Most studies have been carried out with nelfinavir in view of its stronger anticancer efficacy in the preclinical setting. Ongoing phase II trials are primarily in myeloma (in association with bortezomib and lenalomide), glioma (in association with chemoradiation), pancreas (in association with gemcitabine and radiation), lung (in association with radiation with concurrent chemotherapy, i.e., cisplatin and etoposide) and cervical cancer.

The first phase I/II clinical trial of nelfinavir for liposarcomas (NCT00233948) showed that nelfinavir may be an option for the treatment of subjects with unresectable liposarcomas.[46] With the exception of one subject experiencing reversible, grade 3 pancreatitis, no dose-limiting toxicities were observed; these included grade 1 or 2 hematologic toxicities (i.e., anemia and lymphopenia), diarrhea and liver toxicity (i.e., alkaline phosphatase and AST elevation). Clinical benefit was observed in 6 of 20 subjects, which is a promising result given that liposarcomas are relatively resistant to chemotherapy.

A phase I trial of nelfinavir in combination with a fixed dose of cisplatin and escalating doses of gemcitabine in combination with radiation for locally advanced pancreatic cancer showed that Nelfinavir added to chemoradiotherapy was well tolerated.[47] Partial CT responses were observed in 5 of 10 patients (KB: In the previous and following paragraphs you use digits for the numbers of patients) who completed chemoradiotherapy and minor responses were observed in 2 of 10 patients. Of 9 patients assessable by PET, responses were complete in 5 and partial in 2 patients; stable disease was observed in 2 patients. CA19-9 tumor marker levels decreased after therapy in 8 of 9 assessable patients. Therefore, the observed PET/CT and CA19-9 responses support the hypothesis that nelfinavir may increase the chemoradiotherapy effects in borderline or unresectable pancreatic ductal adenocarcinoma.

A phase I trial of the HIV-PI nelfinavir with concurrent chemoradiotherapy for unresectable stage IIIA/IIIB non-small cell lung cancer (NSCLC) showed no dose limiting toxicity at the two tested doses (625 mg PO BID and 1250 mg PO BID).[48] Median follow-up for the 12 evaluable patients was 31.6 months compared to 23.5 months for survivors. The locoregional metabolic response rate was 100%, with 5 of 9 patients (56%) having a complete response on PET/CT obtained 3 months after completion of treatment. This trial suggests that nelfinavir may have a positive effect in NSCLC.[48]

In a Phase I trial of nelfinavir in combination with radiation and capecitabine 825 mg/m2 BID for locally advanced rectal cancer, the recommended phase II dose (RP2D) for

Table 2. Clinical trials of HIV-PIs in cancer

NCT Number	Drug(s)	Conditions	Concurrent therapy	Phase	Enrollment	Start Date
NCT00589056	Nelfinavir	Lung Cancer	radiation	I/II	42	June 2007
NCT00436735	Nelfinavir	Colorectal Cancer/Gastrointestinal Carcinoid Tumor/Head and Neck Cancer/Islet Cell Tumor/Lung Cancer/Metastatic Cancer/Neuroendocrine Carcinoma of the Skin/Ovarian Cancer/Pheochromocytoma/Sarcoma/Unspecified Adult Solid Tumor, Protocol Specific		I	45	September 2006
NCT02080416	Nelfinavir	Non-Hodgkin Lymphoma/Hodgkin Lymphoma/Kaposi Sarcoma/Gastric Cancer/Nasopharyngeal Cancer/EBV/Castleman Disease		0	10	July 2014
NCT01068327	Nelfinavir	Adenocarcinoma of the Pancreas/Stage III Pancreatic Cancer	gemcitabine hydrochloride, leucovorin calcium and fluorouracil	I	46	November 2007
NCT01959672	Nelfinavir	Pancreatic Adenocarcinoma/Resectable Pancreatic Cancer/Stage IA Pancreatic Cancer/Stage IB Pancreatic Cancer/Stage IIA Pancreatic Cancer/Stage IIB Pancreatic Cancer/Stage III Pancreatic Cancer	gemcitabine hydrochloride, leucovorin calcium and fluorouracil, with or without oregovomab followed by stereotactic body radiation	II	66	September 2013
NCT00704600	Nelfinavir	Colorectal Cancer/Colorectal Carcinoma/Colorectal Tumors/Neoplasms, Colorectal		I/II	15	September 2008
NCT01445106	Nelfinavir	Solid Tumors		I	28	December 2006
NCT01485731	Nelfinavir	Cervical Cancer	Cisplatin Chemotherapy With Pelvic Radiation	I	8	January 2012
NCT01079286	Nelfinavir	Renal Cell Cancer/Cancer	Temsirolimus	I	18	June 2009
NCT01065844	Nelfinavir	Carcinoma, Adenoid Cystic/Head and Neck Neoplasms		II	35	October 2009
NCT02024009	Nelfinavir	Pancreatic Neoplasms (Locally Advanced Non-metastatic)	Nab-paclitaxel	I/II	289	March 2016
			Radiation: 60Gy in 30#			
			Radiation: 50.4Gy in 28#			
			Capecitabine			
			Gemcitabine			
NCT01086332	Nelfinavir	Pancreatic Neoplasms	Gemcitabine	I	7	May 2009
NCT01164709	Nelfinavir	Leukemia/Lymphoma/Mature T-cell and Nk-cell Neoplasms/Multiple Myeloma and Plasma Cell Neoplasm	Bortezomib	I	18	July 2010
NCT02363829	Nelfinavir	Uterine Cervix Cancer	Cisplatin	I	6	February 2015
			Pelvic radiation			
NCT01108666	Nelfinavir	Non Small Cell Lung Cancer	Cisplatinum + Etoposide or Carboplatin + Paclitaxel	I	72	March 2010
NCT00915694	Nelfinavir	Brain and Central Nervous System Tumors	Radiation therapy and temozolomide	I	23	39904
NCT01447589	Nelfinavir	Lung Cancer	Radiotherapy	I/II	0	February 2012
NCT01925378	Nelfinavir	Cervical Dysplasia		II	10	July 2012

Table 2. Clinical trials of HIV-PIs in cancer (Continued)

NCT Number	Drug(s)	Conditions	Concurrent therapy	Phase	Enrollment	Start Date
NCT00791336	Nelfinavir	Carcinoma, Non-Small-Cell Lung		II	1	August 2008
NCT00694837	Nelfinavir	Glioblastoma	Temozolomide and Radiotherapy	I	6	March 2009
NCT01020292	Nelfinavir	Glioma	Temozolomide and Radiotherapy	I	31	39904
NCT01555281	Nelfinavir	Multiple Myeloma	Lenalidomide/ Dexamethasone	I/II	32	41000
NCT02207439	Nelfinavir	Stage III, IVa, or IVb Squamous Cell Carcinoma of the Oral Cavity, Oropharynx, Larynx, or Hypopharynx	Chemoradiotherapy	II	28	July 2014
NCT01728779	Nelfinavir	Oligometastases	Stereotactic Body Radiation	II	42	41365
NCT00233948	Nelfinavir	Adult Liposarcoma/Recurrent Adult Soft Tissue Sarcoma/ Stage III Adult Soft Tissue Sarcoma/Stage IV Adult Soft Tissue Sarcoma		I/II	29	March 2006
NCT02188537	Nelfinavir	Myeloma	Bortezomib	II	34	December 2014
			Dexamethasone			
NCT00002185	Nelfinavir	Sarcoma, Kaposi/HIV Infections		II	20	null
NCT01095094	Ritonavir/ Lopinavir	Brain Tumor/Anaplastic Astrocytoma/Anaplastic Ependymoma/Anaplastic Oligodendroglioma/Brain Stem Glioma/Giant Cell Glioblastoma/Glioblastoma/Gliosarcoma/Mixed Glioma		II	19	January 2009
NCT00444379	Ritonavir/ Lopinavir	KS/HIV Infections	Emtricitabine/Tenofovir Efavirenz plus Emtricitabine/Tenofovir.	IV	224	39173
NCT00834457	Ritonavir/ Lopinavir	AIDS-related KS	Abacavir/3TC	II/III	49	June 2007
NCT01009437	Ritonavir	Breast Cancer	Therapeutic conventional surgery	I/II	52	May 2010
NCT01095094	Ritonavir/ Lopinavir	Brain Tumor/Anaplastic Astrocytoma/Anaplastic Ependymoma/Anaplastic Oligodendroglioma/Brain Stem Glioma/Giant Cell Glioblastoma/Glioblastoma/Gliosarcoma/Mixed Glioma		II	19	January 2009
NCT00637637	Indinavir Ritonavir	Cancer	Radiation therapy	II	60	September 2007
NCT00002366	Ritonavir	Sarcoma, Kaposi/HIV Infections		II	null	null
NCT00444379	Ritonavir/ Lopinavir	KS/HIV Infections	Emtricitabine/Tenofovir Efavirenz plus Emtricitabine/Tenofovir	IV	224	39173
NCT00834457	Ritonavir/ Lopinavir	AIDS-related KS	Abacavir/3TC	II/III	49	June 2007
NCT00637637	Indinavir Ritonavir	Cancer	Radiation therapy	II	60	September 2007
NCT01067690	Indinavir	KS	Vinblastina +/− Bleomicina	II	25	June 2008
NCT00362310	Indinavir	Classical KS		II	28	June 2003

nelfinavir was found to be 750 mg BID.[49] Three of 11 patients (27%) had a complete response and 4 of 11 patients (36%) had a major response. These response rates were considerably higher than those reported in trials using a comparable chemoradiation regimen,[50] but further studies are required to demonstrate a significant synergistic effect of nelfinavir.

In another phase II trial of 10 patients with advanced metastatic rectal cancer treated for 7 days with oral nelfinavir (1250 mg bid) and for further 7 days with nelfinavir during pelvic RT (25 Gy/5 fractions/7 days), median tumor cell density decreased from 24.3% at baseline to 9.2% in biopsies taken 7 days after RT. Overall, 5/9 evaluable patients exhibited good tumor regression on MRI assessed by Tumor Regression Grade.[51]

In a Phase I study conducted on newly diagnosed glioblastoma after surgical resection, 21 patients were treated with standard radiotherapy (6,000 cGy to the gross tumor volume), temozolomide (75 mg/m^2 daily) together with daily oral nelfinavir starting 7–10 days prior to chemoradiotherapy continuing for the duration of chemoradiation for 6 weeks.[52] Two doses of nelfinavir were investigated: 625 mg bid and 1,250 mg bid in a cohort escalation design. No dose-limiting toxicity was observed at 625 mg bid. At 1,250 mg bid, 3 dose-limiting episodes of hepatotoxicity and 1 of diarrhea were observed. The maximally tolerated dose was 1,250 mg bid. The percentage of patients with Out Of Field (OOF) recurrences was 14.3, and the Progression Free Survival (PFS) among the three patients with OOF recurrence was more than double the overall mean PFS, suggesting that better local tumor control for a longer period led to increased likelihood of first recurrence elsewhere. Despite these observations, larger cohorts of patients should be tested to assess the efficacy of nelfinavir in association with chemotherapy in glioblastoma patients.

In a trial of 28 patients with refractory cancers (colorectal, SCLC and NSCLC, carcinoid, thyroid, renal, adenoic cystic, sarcoma, head and neck, pancreatic and prostate cancer), oral nelfinavir was generally well tolerated.[53] The Maximal Tolerated Dose (MTD) was 3125 mg twice daily. In an expansion cohort given MTD, 1/11 (9%) evaluable subjects had a confirmed partial response. This plus two minor responses occurred in patients with neuroendocrine tumors of the midgut or pancreatic origin. Thirty-six % of subjects had stable disease for >6 months.[53] In another study, no efficacy was observed in 15 patients with adenoid cystic carcinomas.[54] The median progression-free survival was 5.5 months and no patient achieved a partial or complete response.

In a phase I study of patients with advanced hematologic malignancies, the combination of nelfinavir with the proteasome inhibitor bortezomib showed little or no effect.[55] Of 10 evaluable patients in a dose escalation cohort, three achieved a partial response, four had stable disease for two cycles or more and three suffered progressive disease. In addition, in an exploratory extension cohort with six relapsed, bortezomib-refractory, lenalidomide-resistant myeloma patients treated at the recommended phase II dose (2 $\times$ 2500 mg), three reached a partial response, two a minor response and one had progressive disease. This suggests that nelfinavir may overcome the biological features of proteasome inhibitor resistance, likely by upregulating expression of proteins related to the UPR (such as, PDI, BIP, CHOP and PARP) in peripheral blood mononuclear.

However, an observational study to examine the association between cancer incidence and nelfinavir treatment revealed that the drug was not associated with a lower cancer incidence compared to other protease inhibitor regimens.[56]

Indinavir

Currently, 3 clinical trials on indinavir in cancer are listed on Clinicaltrials.gov: (*i*) a phase II trial, NCT00637637, studying external-beam radiation therapy together with indinavir and ritonavir in patients with brain metastases in comparison to radiation therapy alone; (*ii*) NCT01067690, testing the effects of indinavir in association with vinblastin $\pm$ bleomycin in patients affected by advanced non HIV-associated KS; and (*iii*) NCT00362310, a single group assignment, non-randomized, open-label phase II study of indinavir in non-HIV-related KS. In the latter, 28 patients with early-stage KS (stage I or II, 14 patients) or late-stage KS (stage III or IV, 14 patients) were enrolled.[57] Treatment consisted of 800 mg of indinavir twice daily for 12 months. Adverse events were infrequent and modest, that is, mild-to-moderate asthenia or arthralgia and nonspecific skin manifestations such as erythema, rash, or itching. A favorable effect of treatment was observed in 61.5% of the patients with complete remission in 1 patient, partial regression in 2, improved disease in 5 and stabilization of progressive disease in 8. A non-favorable clinical course was observed in 38.5%, mostly in patients suffering from late-stage KS.

Lopinavir/ritonavir

Lopinavir shows low bioavailability when given alone, while blood levels are significantly increased by low-dose ritonavir.[58] For this reason, the combination of lopinavir/ritonavir is often tested in clinical trials. The NCT00444379 trial has studied whether a protease inhibitor-based antiretroviral regimen (lopinavir/ritonavir 200/50 mg plus emtricitabine/tenofovir 200/300 mg) is more effective than a non-nucleoside reverse transcriptase inhibitor-based antiretroviral regimen (efavirenz 600 mg plus emtricitabine/tenofovir 200/300 mg) promoting the regression of KS tumor burden in persons with AIDS-related KS in Africa. The outcome of this study is currently not available. In addition, it is actually unclear whether in this study the dose was 200/50 mg daily or 400/100 mg BID that is the dose of lopinavir/ritonavir usually used in the antiretroviral regimens.

In another phase II trial, NCT01095094, ritonavir/lopinavir (400 mg/100 mg BID) was tested in 19 patients with progressive or recurrent high-grade gliomas.[59] A complete

response was seen in 1 Patient (5%), 3 (16%) had stable disease as best outcome and 15 (79%) had progressive disease. Six-months progression-free survival was seen in only 2/19 patients (11%) and the study did not meet its primary efficacy endpoint.

Finally, a single-arm, proof-of-concept trial of self-applied topical treatment with lopimune (lopinavir/ritonavir) in 23 women with HPV-related cervical high grade squamous intraepithelial lesions, ISRCTN Registry 48776874, demonstrated a combined positive response in 81.8%, 77.8% of which was confirmed histologically.[60]

Bioavailabity and Toxicity of HIV-PIs

Despite the interest generated from the convincingly emerging anticancer action profile of HIV-PIs, a major concern for therapeutic applications in cancer is their low biological availability and a degree of toxicity. Up to now, darunavir boosted with ritonavir (DRV/r) is the preferred HIV-PI in the US Department of Health and Human Services treatment guidelines for naïve patients, in combination with tenofovir/emtricitabine,[61] and atazanavir boosted with ritonavir and lopinavir boosted with ritonavir are the preferred second-line antiretroviral therapy.[62] However, there is no indication for the use of nelfinavir nor indinavir in HAART.

The main barriers for HIV-PIs absorption[63] are the expression and distribution of different ATP-binding cassette drug transporters in the intestine and the enzyme system-cytochrome 450, mainly the CYP3A4 isoform.[63] Moreover, P-glycoprotein is expressed in a variety of excretory tissues, liver, kidney and at blood-tissue barriers such as the blood-brain barrier, the blood-testis barrier and the placenta.[64] It was found that attained HIV-PIs plasma levels, and the diffusion of the drugs to immune privileged tissues at least partly depends on the same MDRI P-glycoprotein (P-gp) transporters.[65] The substrates of P-glycoprotein and drug-metabolizing enzymes, particularly CYP3A4, overlap and the inhibitors of P-glycoprotein are also effective as inhibitors of CYP3A4.[66] Saquinavir and ritonavir are both substrates and inhibitors of P-glycoprotein. However, ritonavir remarkably enhances saquinavir effectiveness through inhibition of CYP3A4 rather than P-glycoprotein.[67] First-pass liver metabolism mediated by CYP3A4 expression on hepatocytes is one of the key causes of low biological availability of indinavir,[68] nelfinavir[69] and saquinavir.[70] In the blood, most HIV-PIs bind primarily to alpha-1-acid glycoprotein (AGP), which affects tissue delivery and excretion of numerous drugs. It has been shown that the *in vitro* efficacy of HIV-PIs decreases with increased blood levels of AGP.[71] Furthermore, increased levels of AGP abrogate the volume of saquinavir distribution and enhance plasma saquinavir binding in the transgenic mouse model.[71] Thus, drug efficacy is compromised even with higher plasma levels of HIV-PI.[71]

The apical expression of energy dependent efflux pumps as (P-gp) and multidrug resistance protein 2 (MRP2) at the vascular endothelial cells determines tissue delivering of HPIs.[72] In contrast to P-glycoprotein and MRP2, MRP1, −3 and −5 and Bcrp1 are not classified as efficient transporters of saquinavir, ritonavir and indinavir.[73] Hence, the *in vivo* effects of HIV-PIs can be improved by combined treatment with MRP2 inhibitors. In addition, nelfinavir is both an inhibitor and a substrate of MRP4.[74] Together with P-glycoprotein and MRP2 efflux transporters, influx transporter OATP-A is also included in transport and excretion of HIV-PIs. This is especially important in liver-mediated detoxication activities and saquinavir excretion into bile.[75]

By acting as cytochrome P-450 inhibitors, HIV-PIs are able to significantly modify the pharmacokinetics of other drugs with ritonavir and saquinavir being the most and less potent, respectively. The effects on cytochrome P-450 are augmented when two HIV-PIs are given simultaneously.[1] Ritonavir inhibits hepatic metabolism of saquinavir, increasing its plasma levels 20- to 30-fold. Nelfinavir increases the area under the plasma-concentration–time curve by 392% for saquinavir and by 51% for indinavir. Indinavir increases the area under the curve of saquinavir by about 5-fold.[1]

Even if HIV-PIs are created as peptide-mimetics with highly specific affinity to the HIV protease binding site, numerous mammalian proteins are affected directly or consequently by their action. This results in various toxic events that often follow long term treatment with most members of HIV-PIs. These include gastrointestinal, renal and hepatic adverse effects. Thus, nausea, vomiting and abdominal pain are frequently associated with ritonavir, especially during the first few weeks of therapy, and diarrhea is the dose-limiting side effect of nelfinavir. Serum aminotransferase elevation have been reported, but hepatitis is uncommon. Reversible unconjugated hyperbilirubinemia is frequent in patients taking indinavir but rarely associated with high serum aminotransferase concentrations or overt liver disease. Nephrolithiasis and crystalluria are the most important side effect of indinavir and can occur within a few days after start of therapy. Several cases of hemolytic anemia have also been reported with the use of indinavir.[1]

Other common side effects include metabolic syndromes such as dyslipidemia, insulin-resistance and lipodystrophy. Interestingly, no significant differences have been observed between HIV-PIs monotherapy and the combination of protease inhibitors with the HIV integrase inhibitor, raltegravir, nor nucleoside reverse transcriptase inhibitors, suggesting that HIV-PIs may be mainly responsible for the adverse effects.[14]

Lipodystrofy induced by HIV-PIs has been associated with the effects on adipocyte transcription factors such as peroxisome proliferators activated receptor γ (PPAR-γ) and SREBP-1. In particular, a >2-fold increase in SREBP-1 expression has been found in HIV infected individuals treated with ritonavir.[76] However, data on lipodystrophy are conflicting and some longitudinal studies have failed to demonstrate the association with HIV-PIs.[77]

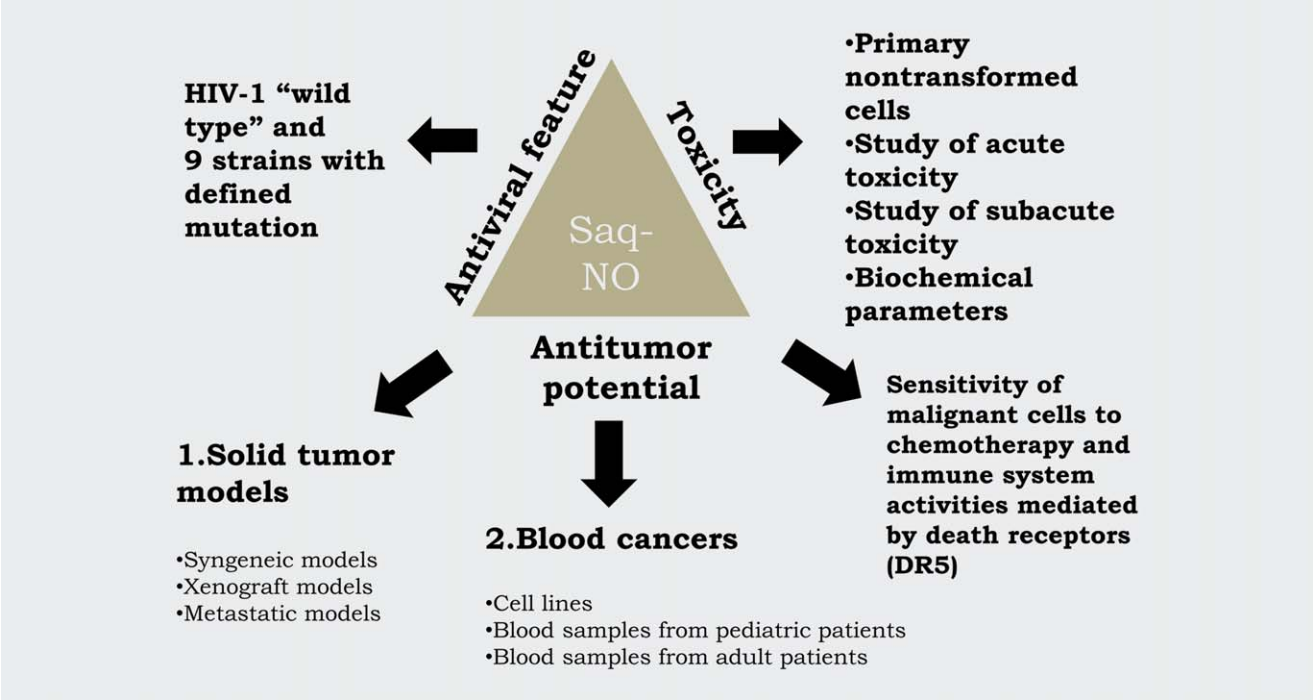

Figure 1. Saq-NO features. [Color figure can be viewed at wileyonlinelibrary.com]

Insulin resistance may be induced by HIV-PIs via multiple mechanisms. HIV PIs have been shown to inhibit the release of insulin by pancreatic beta-cells,[78] and to decrease the response to insulin of skeletal muscle cells, adipocytes and hepatocytes, likely by inhibition of Akt and protein kinase C signaling and through direct binding to the glucose transporters, Glut1 and Glut4.[79–81]

Preclinical Anticancer Effects of Novel HIV-PIs

Analogs of HIV-PIs

The development of new drugs is expensive and time consuming. Novel applications of drugs already approved for other indications are therefore important especially if supported by clear-cut preclinical and clinical data. In this regard, the repeatedly demonstrated anticancer activity of HIV-PIs is promising, but the above-mentioned toxicity along with nonoptimal pharmacokinetic properties and an overall modest therapeutic potency has propelled several groups to generate derivatives of HIV-PIs for anticancer use through modifications such as attachment of different moieties, ligands and transporters.

You *et al.* has synthesized a new indinavir analogue with important anticancer activity, CH05-10.[82] This drug achieved similar cytotoxity against leukaemia, melanoma, ovarian and prostate cancer cell lines as nelfinavir, but at lower concentrations. It induced cell cycle arrest in G1 and caused caspase-dependent apoptosis, but also caspase-independent death via the induction of ERS and UPR.

Using a different attempt, Singh and coworkers created saquinavir-loaded folic acid conjugated PEGylated and non-PEGylated poly(D,L-lactide-*co*-glycolide) (PLGA) nanoparticles (NPs) (Saq-Fol-PEG-PLGA and Saq-Fol-PLGA), which were tested on human prostate and breast cancer cell lines (ref?). Effective concentrations were 56 ± 0.60 and 58 ± 0.80 w/v for Saq-Fol-PEG-PLGA and Saq-PLGA NPs, respectively. Saq-Fol-PEG-PLGA displayed elevated cytotoxicity, cellular uptake and high selectivity toward the malignant cells. Saq-Fol-PEG-PLGA NPs had enhanced anticancer potential in comparison to non-targeted Saq-PLGA NPs.[83]

NO-modified HIV-PIs

During the last 10 years, our group has been committed to generate NO-derivatives of HIV-PIs. The rationale behind this relied on observations that hybridization with NO promoted anticancer effects of nonsteroid antiinflammatory drugs (NSAID). For example, NO-acethylsalicylic acid and other NO-NSAIDs exhibit anticancer activity in a wide range of cancer cell lines and in *in vivo* models,[84–95] and these compounds are invariably more potent than the corresponding NSAID analogs. The mechanism(s) of action of NO-NSAIDs as cancer chemotherapeutic agents is likely to be multifactorial as they inhibit tumor cell growth, induce apoptosis and exhibit antiangiogenic and antimetastatic activity.[84–95] Along this line of research we have also demonstrated that hybridization of the immunomodulatory compound GIT-27 with NO endowed this compound with unique chemotherapeutic properties *in vitro* and *in vivo*, features that were not seen with the parental compound.[96] Hence, we hypothesized that NO-hybridization of HIV-PIs may enhance

their anticancer actions allowing lower dosing and reduced side effects.

The modality of NO hybridization chosen consisted of covalent linking of the NO moieties to the parental molecule by covalent linking with an OH group of the parental molecule.[97] The HIV-PIs most suitable for this NO-hybridization were saquinavir, lopinavir and ritonavir.

This line of research was initially carried out at GaNiAl Immunotherapeutics (Wilmington, DE) and subsequently at OncoNox (Copenhagen, Denmark). The data generated indicate that Saq-NO is a new chemical entity with unique behavior in a variety of experimental systems *in vitro* and *in vivo* (Fig. 1). Although Saq-NO retained an antiretroviral action superimposable to that of saquinavir, the toxicity of Saq-NO was significantly lower than that of saquinavir.[103] Therefore, in addition to non-toxicity toward primary astrocytes and fibroblasts *in vitro*, there was no lethality after exposure of animals to Saq-NO at a dose corresponding to the lethal dose of saquinavir.[97] However, anticancer activity of the modified drug compared to saquinavir was elevated *in vitro* (Table 3).[97,100–104] Independent confirmation of these findings was achieved using NCI screening of 60 cell lines (Fig. 2). Furthermore, the *in vitro* data were substantiated by experiments showing Saq-NO anticancer activity in syngeneic and xenograft models of melanoma, prostate and colon cancers (Table 4).

Table 3. Cell lines sensitive to Saq-NO

Origin	Cell line	Type	References
Mouse	B16	Melanoma	97
Mouse	CT26CL25	Colon carcinoma	98
Rat	C6	Glioma	97
human	PC-3	Prostate adenocarcinoma	99
human	LnCap	Prostate carcinoma	104
human	HCC1419	Breast carcinoma	97
human	BT20	Breast carcinoma	97
human	A375	Melanoma	100,101
human	HeLa	Cervical adenocarcinoma	97
human	HCT116	Colorectal carcinoma	98
human	HL60	Acute promyelocytic leukemia	102
human	Jurkat	Acute T cell leukemia	102
human	Raji	Burkitt's lymphoma	102
human	K562	Chronic myelogenous leukemia	102

It is important to note that the *in vivo* experiments were carried out under a "therapeutic regimen"; that is, postponing Saq-NO treatment until the tumor was palpable. The improved anticancer action of Saq-NO over the parent compound cannot be ascribed to cytotoxicity of released NO since only insignificant quantities were detected in cells after therapy. In addition, the therapeutic effect cannot be simulated by independent application of saquinavir and the NO donor, DETA NONOate, underlining the unique anticancer property of the newly developed drug.[101]

In a manner similar to that described for HIV-PIs, induction of apoptosis by Saq-NO was always connected with some cellular specificity like p53 deficiency or constitutive expression of iNOS.[99,100,102] In other cancer cell lines, the inhibition of proliferation was accompanied by differentiation and transdifferentiation of the tumor cells toward their normal counterparts. Thus, Saq-NO induced changes to cells bearing markers of their ancestors or even embryonic progenitors was noted in the case of astrocytoma and melanoma, and this effect was not observed with saquinavir.[97]

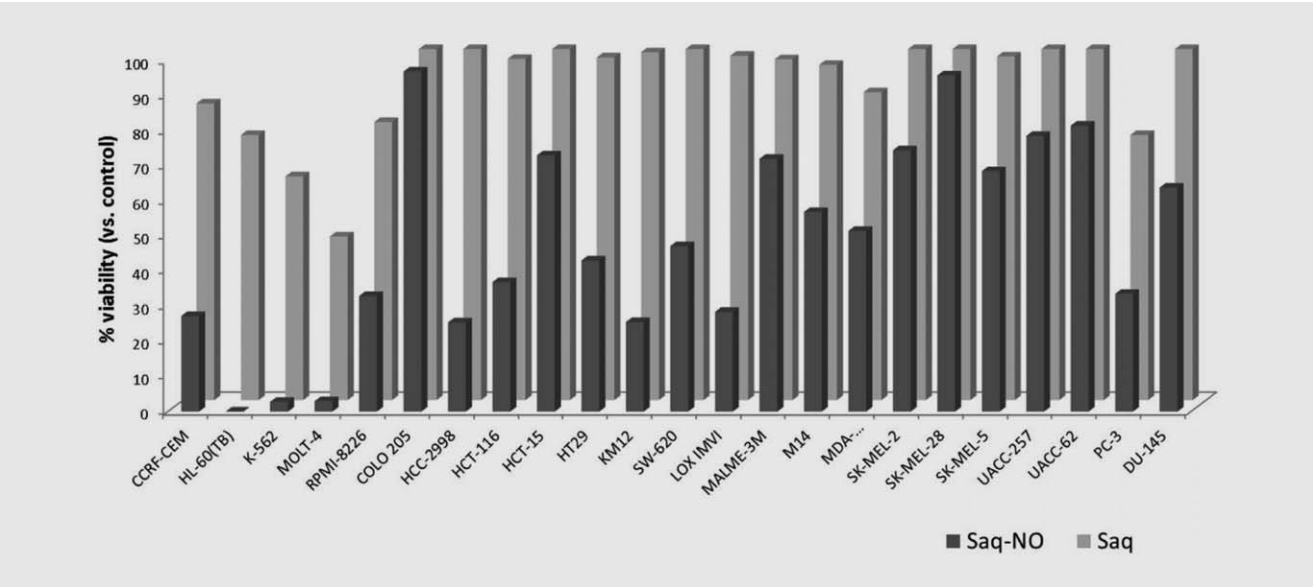

Figure 2. NCI screening of Saq-NO anticancer effect.

Table 4. *In vivo* tumor model

Tumor	Dose	Start of treatment	Duration of treatment	References
B16	10 mg/kg	10 Days after t.i.	15 Consecutive days	83
PC3	10 mg/kg	23 Days after t.i.	19 Consecutive days	90
LnCap	10 mg/kg	23 Days after t.i.	15 Consecutive days	88
CT26CL25	10 mg/kg	14 Days after t.i.	14 Consecutive days	87
A375	10 mg/kg	21 Days after t.i.	24 Consecutive days	85

One of the major pathways involved in the anticancer action of saquinavir and other HIV-PIs is the capacity to downregulate the PI3-Akt-mTOR axis, and this effect may be related with numerous toxic effects accompanying their therapeutic use.[2] In contrast to saquinavir, Saq-NO transiently activates the upstream part of this pathway.[97,100] As inhibition of Akt is responsible for many side effects of saquinavir, different regulation of Akt by Saq-NO could be connected with a loss of general toxicity.[100] Further analysis of molecules involved in the downstream segment of this signaling pathway revealed an inhibitory action of Saq-NO, but not of saquinavir, on p70S6K.[102,105] Indeed, compromised activity of p70S6K by Saq-NO influenced numerous cell activities, for example protein synthesis, cytoskeletal rearrangements, proliferation and cell survival. Sensitization of malignant cells to apoptosis triggered both by chemotherapeutic agents and by tumor necrosis factor-related apoptosis inducing ligand, a product of many cell types, may also be secondary to inhibition of S6K1 function. A spectrum of S6K dependent proteins disturbed by Saq-NO are intracellular caspase inhibitors, xIAP and FLIP, as well as S6 protein responsible for transcription of oligopyrimidin mRNAs. Thus, malignant cells exposed to Saq-NO undergo phenotypic transformation with loss of malignant properties (dividing, migratory and invasive potentials) and acquisition of apoptotic-prone phenotype.[100] In addition, Saq-NO may potentiate the recognition and killing of cancer cells by the immune system as the compound decreased expression of DR4/DR5 repressor YY1 in cells whose vitality is controlled by NO.[100]

Apart from the intracellular events triggered by Saq-NO, chemosensitizing properties may be ascribed to inhibition of p-gp, MRP-1 and BCRP-1.[106] This would make it a candidate for treatment of multidrug-resistant tumors. Furthermore, Saq-NO sensitized P-gp- or MRP1-expressing cancer cells to chemotherapy more potently then saquinavir, whereas BCRP1-expressing cells were equally sensitized by both substances. It was also verified that Saq-NO is a substrate of P-gp as well as of MRP1. Accordingly, Saq-NO may prove valuable for combined treatment of multidrug-resistant tumors.[106]

Few data have been generated on the anticancer potential of other NO-derived HIV-PIs, for example lopinavir-NO and ritonavir-NO. A recent article, however, demonstrated that lopinavir-NO had a 2–4 fold stronger anticancer action on blood cancer cells than its parent compound.[102]

Conclusions

Increasing preclinical and clinical evidence support the potential of HIV-PIs as antineoplastic drugs, with nelfinavir being the most potent. Despite the lack of a unique mechanism of action for HIV-PIs, the antitumor effect seems to be related, in a HIV-PI-dependent manner, to MMP-inhibition, ERS induction, proteasome activity inhibition, AKT phosphorylation and angiogenesis inhibition. It is likely that additional mechanisms of action will be identified. These data make the HIV-PIs promising candidates for cancer therapeutics, also in consideration of the knowledge of their toxicity profile, pharmacokinetics and metabolism and drug interactions. Novel analogs and derivatives of HIV-PIs have been developed, and promising data come from NO-hybridized HIV-PIs, such as saquinavir. This led to the generation of Saq-NO that, while retaining an antiretroviral effect superimposable to that of the parental compound, showed lower toxicity than that of saquinavir and a significantly higher antineoplastic effect. In conclusion, we believe that the present data warrant additional studies aimed at evaluating the impact of NO-hybridization on the chemotherapeutic profile of other HIV-PIs with anticancer potential.

Conflict of Interest Disclosure

Ferdinando Nicoletti is cofounder and shareholder of OncoNox

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