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Inhibitors of the apurinic/aprimidinic endonuclease 1 (APE1)/nucleophosmin (NPM1) interaction that display anti-tumor properties

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Abstract

The apurinic/aprimidinic endonuclease 1 (APE1) is a protein central to the base excision DNA repair pathway and operates in the modulation of gene expression through redox-dependent and independent mechanisms. Aberrant expression and localization of APE1 in tumors are recurrent hallmarks of aggressiveness and resistance to therapy. We identified and characterized the molecular association between APE1 and nucleophosmin (NPM1), a multifunctional protein involved in the preservation of genome stability and rRNA maturation. This protein-protein interaction modulates subcellular localization and endonuclease activity of APE1. Moreover, we reported a correlation between APE1 and NPM1 expression levels in ovarian cancer, with NPM1 overexpression being a marker of poor prognosis. These observations suggest that tumors that display an augmented APE1/NPM1 association may exhibit increased aggressiveness and resistance. Therefore, targeting the APE1/NPM1 interaction might represent an innovative strategy for the development of anticancer drugs, as tumor cells relying on higher levels of APE1 and NPM1 for proliferation and survival may be more sensitive than untransformed cells.

We set up a chemiluminescence-based high-throughput screening assay in order to find small molecules able to interfere with the APE1/NPM1 interaction. This screening led to the identification of a set of bioactive compounds that impair the APE1/NPM1 association in living

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cells. Interestingly, some of these molecules display anti-proliferative activity and sensitize cells to therapeutically relevant genotoxins. Given the prognostic significance of APE1 and NPM1, these compounds might prove effective in the treatment of tumors that show abundant levels of both proteins, such as ovarian or hepatic carcinomas.

Keywords

APE1; NPM1; protein/protein interaction; small molecule; combination therapy

Introduction

Targeting DNA repair pathways to improve tumor therapy is currently one of the most active topics in cancer research. Many compounds targeting different DNA repair components are currently undergoing clinical and pre-clinical investigation as promising molecules that display either a selective cancer killing action or, more often, that improve sensitivity to traditional therapy [1–3].

The human apurinic/apyrimidinic endonuclease 1 (APE1) is a pivotal DNA repair protein, being a central enzyme to the base excision repair (BER) pathway. As the main abasic endonuclease in mammalian cells, this protein is essential for embryonic development [4, 5]. In addition, APE1 acts as a master regulator of cellular transcription, by modulating in a redox-dependent and independent fashion the DNA binding activity of several cancer-related transcription factors (including NF- κ B, Egr-1, p53, HIF-1 α among others) [6]. Aberrant subcellular localization, expression levels and post-translational modification patterns of APE1 have been linked to increased tumor aggressiveness and decreased differentiation, as well as to the onset of chemo- and radio-resistance in different kinds of cancer [7–12]. In light of the association between APE1 and cancer, several laboratories over the last decade have developed strategies to target either its endonuclease activity or its redox function by means of small molecule inhibitors [3, 13–15]. Since APE1 is a ubiquitous protein [10], it is not clear whether these approaches could achieve specificity of action in the context of a systemic administration of the APE1 inhibitor.

We previously reported and characterized the molecular association between APE1 and nucleophosmin (NPM1) [16–18], a nucleolar phosphoprotein involved in tumorigenesis, either as a proto-oncogene or as a tumor suppressor, in a context-dependent manner [19, 20]. The interaction with NPM1 modulates several functions of APE1: it promotes APE1's accumulation within nucleoli, stimulates its endonuclease activity, and, likely, regulates its post-translational modifications and protein interaction network, by masking the unstructured N-terminal domain of the protein [8, 16, 21]. Very recently, we showed that NPM1 is involved in the functional modulation of the BER pathway in cells through direct interaction with APE1 and stimulation of its AP-endonuclease activity [22]. Accordingly, the occurrence of an aberrant cytoplasmic APE1/NPM1 association, observed in acute myeloid leukemia (AML) patients bearing a NPM1 mutation (NPM1c+) [20, 23], leads to an impairment of the BER pathway and to an increased sensitivity to genotoxins [22]. Moreover, the expression of an APE1 mutant unable to stably interact with NPM1 is linked

to a strong reduction in the rate of cellular proliferation [21]. Altogether, these observations suggest that a functional APE1/NPM1 interaction plays a pivotal role in tumor cell proliferation and cell response to genotoxins.

NPM1 overexpression is considered a prognostic marker of recurrence and progression in solid tumors [19, 24, 25]. Furthermore, altered expression of APE1 has been linked to progression of hepatocellular and ovarian carcinomas [7, 9]. Interestingly, in ovarian cancer specimens [25], as well as in hepatic carcinoma cell lines (unpublished data), we detected a positive correlation between NPM1 and APE1 expression levels and the aggressiveness of the malignant phenotype, pointing to these proteins as negative prognostic markers in these pathologies. Such observations suggest that ovarian and hepatic tumors expressing higher amounts of APE1 and NPM1 might display an increased APE1/NPM1 interaction, which may positively impact on tumor cell proliferation and anticancer agent resistance. Interfering with the APE1/NPM1 association, therefore, might prove effective in directly targeting tumor cell proliferation rate and/or in sensitizing them to DNA damaging agents. In addition, this approach might prove more specific and efficient toward cancer cells over-expressing both APE1 and NPM1, overcoming potential systemic toxicity problems.

Here, we describe the discovery, through high-throughput screening (HTS), of small molecules able to interfere with the APE1/NPM1 association. We made use of the AlphaScreen® technology to screen several commercially available small molecule libraries in order to identify, for the first time, a set of molecules that target the APE1/NPM1 interaction. Among the positive hits, we detected known bioactive compounds with novel interesting anti-tumor properties, such as genotoxin-sensitizing and anti-proliferative activities. This study opens new perspectives to target cancer cell proliferation and therapy resistance, while also providing new tools to investigate thoroughly the biological relevance of the APE1/NPM1 association in experimental models and during tumorigenesis.

Materials and Methods

Pilot libraries of bioactive compounds

The screening collection included the following libraries with number of compounds in parentheses: FDA Pharmaceutical Collections (2,816), MicroSource Spectrum collection (1,408), Tocris/TimTec (1,395), and bioactive compounds from Sigma Aldrich LOPAC1280 (1,280), FDA-Tocris-KU-DP (1,376), Prestwick (1,120), BU-GP-BioMol (1,302), Kinacore (2,037) and NCGC chemistry analogues.

AlphaScreen®-based high-throughput screening assay

Assay buffer consisted of 20 mM potassium phosphate pH 7.0, 50 mM NaCl, 5 mM MgCl₂, 0.01% Tween-20 and 2 mM DTT. Microplates used were 384- or 1,536-well white solid-bottom type from Greiner Bio-One (Monroe, NC). Glutathione S-transferase-APE1 fusion protein (GST-APE1) and hexahistidine-labeled NPM1 (His₆-NPM1) proteins were expressed and purified to homogeneity as described previously [16]. AlphaScreen® detection was performed with PerkinElmer Glutathione S-Transferase (GST) and Histidine (Nickel Chelate) Detection Kits (Waltham, MA).

Protein-protein interaction assessment for GST-APE1 and His₍₆₎Tag-NPM1 was initially conducted in 384-well plates. The optimized assay was further miniaturized in 1,536-well plates where, briefly, 3 µl of GST-APE1 (125 nM final) was dispensed by BioRAPTR (Beckman Coulter, Fullerton, CA) flying reagent dispenser, followed by addition of 23 nl of DMSO solution of compound library members (final DMSO concentration was 0.7% [v/v]) achieved by a Kalypsys pintool [26]; after compound addition, 1 µl of His₍₆₎Tag-NPM1 (200 nM final) was dispensed and the assay plates were incubated for 20 minutes at room temperature. For detection, 1 µl of 20 µg/ml Glutathione donor/Ni²⁺ chelate acceptor AlphaScreen® bead mix was added. Plates were briefly centrifuged and incubated for 20 minutes at room temperature. The AlphaScreen® chemiluminescence signal was measured with an EnVision multilabel plate reader (PerkinElmer) equipped with a 1,536 Plate HTS AlphaScreen® aperture (80 ms excitation time, 240 ms measurement time). The signal was compared with that of DMSO-containing control samples; importantly, DMSO had a negligible effect on the assay signal.

Cell culture, chemicals and viability assays

HeLa, Huh7 and MCF7 cells (from ATCC) were grown in DMEM (Invitrogen, Milan, Italy). TOV-112D, HCC70 and Ovar5 cells (from ATCC) were cultured in RPMI 1640 (EuroClone, Milan, Italy), respectively. JHH6 cells (from Health Science Research Resources Bank) [27] were cultured in William's Medium (Sigma, Milan, Italy). OCI/AML cells were obtained and cultured as previously described [22]. Media were supplemented with 10% (v/v) fetal bovine serum (FBS) (EuroClone), 100 U/ml penicillin, and 100 µg/ml streptomycin sulphate. Screening compounds were dissolved in DMSO as 10 mM stocks. MMS was from Sigma-Aldrich and bleomycin sulphate was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

For viability measurements $4-12 \times 10^3$ cells were seeded in 96-well plates; 24 hours later cells were treated with the indicated compound and cell viability was assessed at the indicated time points through the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay – Promega, Milan, Italy) as per manufacturer's instructions. For MMS-combined treatments cells were pre-treated with the selected protein-protein interaction inhibitor as indicated, incubation was then carried out for further 8 hours with increasing amount of MMS, in presence of unchanged inhibitor concentration. For combined treatments with bleomycin 3.0×10^4 cells were seeded into 12-well plates; 24 hours later cells were pre-treated for 8 hours with the APE1/NPM1 inhibitors at the indicated concentration and then incubation was carried out for 1 hour in presence of bleomycin and unchanged compound concentration. Medium was replaced and viability measured 48 hours later by Trypan Blue exclusion. All viability assays were performed in triplicate or quadruplicate and reproduced at least twice in independent experimental sessions.

Immuno-fluorescence and Proximity Ligation Assay (PLA)

Immuno-fluorescence procedures were carried out as described earlier [21]. To monitor the interaction between APE1 and NPM1 in living cells, we used the *in situ* Proximity Ligation Assay kit (Olink Bioscience, Uppsala, Sweden). This assay detects stable, as well as transient interactions by means of a pair of antibodies against the target proteins; short

oligonucleotides linked to the antibodies allow a rolling cycle amplification-based detection of the protein-protein interaction. The signal is visualized through hybridization of fluorescent probes to the amplified oligonucleotides and appears as bright spots that are readily detected through confocal microscopy [28, 29]. HeLa cells stably expressing a FLAG-tagged form of APE1 [21] were seeded on glass slides, treated with the selected compounds, fixed with 4% (w/v) paraformaldehyde immediately after the treatment and incubated with a FITC-conjugated mouse anti-FLAG antibody (1:200 - Sigma) for 3h at 37°C. Cells were then incubated with a rabbit anti-NPM1 (1:200 – Abcam, Cambridge, UK) overnight at 4°C. PLA was performed following manufacturer's instructions. Technical controls, represented by the omission of either the anti-NPM1 or the anti-FLAG primary antibodies, resulted in the complete loss of PLA signal. Determination and scoring of PLA signals was performed using a Leica TCS SP laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 488-nm argon laser, a 543-nm HeNe laser, and a 63× oil objective (HCX PL APO 63× Leica). At least 35 randomly selected cells per condition were analyzed by sectioning the whole cell height into six focal stacks, which were averaged and combined into a single image. This procedure allowed us to detect the PLA signals present throughout the cell, regardless of their subcellular localization. PLA-spots present in each single cell were then scored using the BlobFinder software (Center for Image Analysis, Uppsala University, Uppsala, Sweden); anti-FLAG staining for APE1 was used to identify cell nuclei, allowing us to distinguish between nuclear and cytoplasmic interaction signals.

Surface Plasmon Resonance (SPR) Experiments

Real time binding assays were performed on a Biacore T-100 Surface Plasmon Resonance (SPR) instrument (GE Healthcare, Milan, Italy). Recombinant APE1, APE1 N 33, APE1 KA and NPM1 were immobilized at similar immobilization levels (~2600, 2400, 2400 and 2800 RU, respectively) on a CM5 Biacore sensor chip in 10 mM sodium acetate, pH 5.5, by using the EDC/NHS chemistry, with a flow rate of 5 µl/min and an injection time of 7 min, as previously described [16]. Binding assays were carried out by injecting 100 µl of analyte, at 60 µl/min, with HBS (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, pH 7.4), 0.1 mM TCEP, 10% (v/v) DMSO as running-buffer. The BIAevaluation analysis package (version 4.1, GE Healthcare), was used to subtract the signal of the reference channel.

In vivo assessment of the APE1 redox activity

The APE1 redox function was assessed as described in [27]. Briefly, 1.1×10^4 JHH6 hepatocarcinoma cells were seeded in 96-well plates, 24 hours later cells were co-transfected with 78.4 ng of pIL-8 (interleukin 8 promoter-driven firefly luciferase reporter), 1.6 ng of pRL-CMV (*Renilla* reporter, as a reference for transfection efficiency) and 120 ng of pUC9 carrier plasmid, using the Lipofectamine®2000 Reagent (Invitrogen) as per manufacturer's instructions. One day after transfection cells were pre-treated with the selected APE1/NPM1 inhibitor (10 µM for 5 hours), or with (2E)-3-[5-(2,3-dimethoxy-6-methyl 1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid (E3330, Sigma) as positive control (100 µM for 4 hours) in serum-free medium and subsequently challenged with 2000 U/ml TNF-α (PeproTech Inc., Rocky Hill, NJ) for further 3 hours. The activity of luciferases was

eventually measured using the Dual-Glo® Luciferase Assay System (Promega) using a Modulus™ II Microplate Multimode Reader (Turner Biosystems Inc. Sunnyvale, CA).

Colony formation assays and assessment of the cellular growth rate

For colony formation assays 2×10^2 HeLa cells were plated onto 6-well plates; 24 hours later cells were exposed to the selected compound(s) and cells were allowed to grow until formation of visible colonies (9–11 days) and stained as described earlier [17]. To test the effect of chronic exposure to the APE1/NPM1 inhibitors, medium with the indicated amount of fresh compound was replaced every three days. In the experiments combining bleomycin and the APE1/NPM1 inhibitors, the indicated amount of bleomycin was mixed with 10 μ M inhibitor and treatment was carried out for 1 hour, medium was replaced and cells were allowed to form colonies in fresh medium.

For cellular growth rate measurements, 2×10^4 cells were seeded in 24-well plates, treated 24 hours later and counted at the indicated time points using a coulter counter (Beckman Coulter).

DNA damage accumulation measurements

Genomic DNA was isolated from 1×10^6 HeLa cells by using the Get *pure*DNA Kit (Dojindo, Rockville, MD) and AP-sites content was measured by using the DNA Damage Quantification Kit (Dojindo) as per manufacturer's indications. Briefly, 1 μ g of genomic DNA was labeled with a biotinylated aldehyde reactive probe (ARP) for 1 hour at 37°C, and ARP-DNA was purified following the manufacturer's instructions. The amount of labeled ARP-DNA was then quantified through a colorimetric reaction and eventually measured using a calibration curve provided with the kit.

Alkali comet assay was carried out essentially as described in [30]. Briefly, HeLa cells were exposed to the indicated concentrations of the APE1/NPM1 inhibitors for 16 hours and washed twice with ice-cold PBS. Approximately 3×10^3 cells were embedded on slides in 0.5% (w/v) low melting point agarose (Cambrex Corporate, East Rutherford, NJ) in PBS. The slides were placed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0, 1% (v/v) Triton X-100) for 1 hour at 4°C, and washed in cold 0.4 M Tris (pH 7.4). Next, the slides were incubated in alkali solution (300 mM NaOH, 1 mM EDTA, final pH 13.0) for 30 minutes at 4°C and then electrophoresed horizontally for 30 minutes at 25 V, 350 mA at 4°C. Slides were briefly neutralized with 0.4 M Tris (pH 7.4), stained with ethidium bromide (2 μ g/ml) and viewed on a Zeiss Axiovert 200 M fluorescent microscope (Zeiss, Thornwood, NY). The analysis of the comet tail was carried out using the Comet Assay IV software (Perceptive Instruments, Suffolk, UK) to determine the Olive tail moment. At least 100 cells per experimental condition were scored.

rRNA maturation kinetics

rRNA processing was monitored as described in [31] with minor modifications. In brief, 5×10^5 HeLa cells were treated with the indicated inhibitors. For metabolic labeling cells were trypsinized and immediately phosphate-depleted under rocking at 37°C for 1 hour in presence of unchanged drug concentrations, by incubation in phosphate-free DMEM

supplemented with 10% (v/v) dialyzed FBS (Invitrogen). Medium was then replaced with phosphate-free DMEM supplemented with 10% (v/v) dialyzed FBS containing 15 $\mu\text{Ci/ml}$ [^{32}P]orthophosphate (Perkin Elmer, Milan, Italy) and cells were labeled for 1 hour. Medium was again replaced with normal DMEM supplemented with 10% (v/v) FBS in presence of unchanged drug concentrations and total RNA was isolated after 2 hours using the Trizol® Reagent (Invitrogen). RNA was separated on an agarose-formaldehyde gel loading the same amount of radioactivity per lane. Gels were vacuum-dried and subjected to autoradiography.

AP-site incision assays

APE1 endonuclease activity was monitored using purified recombinant APE1. Enzymatic reactions were carried out in a final volume of 10 μl using 2.3 fmol of protein in a buffer containing 50 mM HEPES pH 7.5, 50 mM KCl, 10 mM MgCl_2 , BSA 1 $\mu\text{g}/\mu\text{l}$ and 1 mM DTT; samples were pre-incubated for 15 minutes at 37°C with 230 pmol of the selected inhibitors. Reactions were started by adding 100 nM of double stranded abasic DNA substrate (obtained by annealing a DY-782-labeled oligonucleotide 5'-CTTGGAAGTGGATGTCGGCAGCAGGATACAGGAGCA-3' (Dyomics), where F indicates a tetrahydrofuran residue, with the complementary sequence 5'-TGCTCCTGTATCCGCTGTGCCGACATCCAGTTCCAAG-3') and incubated at 37°C for the indicated time points. Reactions were halted by addition of formamide buffer (96% formamide, 10 mM EDTA and gel Loading Buffer 6 $\times$ (Fermentas)), separated onto a 20% (w/v) denaturing polyacrylamide gel and analyzed on an Odyssey CLx scanner (Li-Cor Biosciences). The percentage of substrate converted to product was determined using the ImageStudio software (Li-Cor Biosciences).

Statistical analyses

Statistical analyses were performed by using the Student's t test. $p < 0.05$ was considered as statistically significant.

Results

An AlphaScreen®-based high-throughput screening assay for the identification of low molecular weight APE1/NPM1 interaction disruptors

Screening for low molecular weight compounds that target a protein-protein interaction interface is a challenging task. In order to find molecules able to impair the APE1/NPM1 association, we exploited the AlphaScreen® technology using recombinant purified full length GST-tagged APE1 and His₍₆₎-tagged NPM1. The AlphaScreen® assay relies on a proximity-based reporting system used to measure the binding between two cognate partners. Laser excitation at 680 nm of a colloidal-size donor bead releases a flow of singlet oxygen (Fig. 1A). Acceptor beads in close proximity (<200 nm) utilize this singlet oxygen to generate a chemiluminescence signal emitting in the 520–620 nm range. Here, we have configured an AlphaScreen® assay by using glutathione-coated donor beads and Ni²⁺ chelate-coated acceptor beads to measure binding of GST-APE1 to His₍₆₎-NPM1. To search for compounds with the ability to disrupt the APE1/NPM1 interaction, we screened a pilot set of libraries composed of 12,700 small molecules at a seven-concentration dilution series (3.7 nM to 57 μM). An excellent Z' score (>0.65) was maintained throughout the screen

(Fig. 1B). Compounds that showed inhibitory activity in the primary screen were rescreened for confirmation. To eliminate false positives, we used a counterscreen assay to measure the binding AlphaScreen® beads to a GST-His₍₆₎ conjugate, which served as a recognition moiety for both donor and acceptor beads outside the context of the APE1/NPM1 interaction. With this approach, we confirmed 58 compounds as true hits (chosen based on availability, Supplementary Table 1), which were used in further downstream analyses. Representative dose-response curves for a subset of positive compounds are reported in Figure 1C.

Secondary validation assays for the selection of molecules able to impair the APE1/NPM1 interaction in living cells

The positive hits from the primary screening analyses were subjected to a panel of orthogonal cell-based secondary validation assays in order to focus on those molecules able to impair the APE1/NPM1 association in living cells. HeLa cells are a well characterized cell model that has been widely used by our and other laboratories to study APE1 biology [32–35] and to screen for APE1 inhibitors [36, 37]. Thus we used them as a general model to probe the activity of the putative APE1/NPM1 inhibitors. All 58 of the initial positive compounds was preliminarily tested over a wide range of doses (0.1 – 100 µM) and time points (4 – 24 hours) assessing cell viability through the MTS assay. These initial experiments allowed us to estimate the range of solubility in the cell medium (evaluating particle deposition through a phase contrast microscope) and the cytotoxicity for each compound (not shown).

As the interaction with NPM1 is known to modulate the subcellular localization of APE1 [21, 22], we used an immuno-fluorescence-based approach to detect any change in subcellular distribution of APE1 upon treatment with the molecules. Timing and dosage of the treatments were selected on the basis of the individual toxicity and solubility of every compound. In order to reduce the likelihood of confounding effects, for each of the 58 putative inhibitors, we tested at least two conditions (time and/or dosage) at which cell viability was affected by less than 50%. Treatment with the molecules affected the APE1 subcellular localization in different ways (Supplementary Table 1): a number of compounds (e.g. SB 206553, spiclomazine, etc.) increased the cytoplasmic localization of APE1, while others (e.g. ZM 241385, troglitazone, fiduxosin) led to a reduction of the APE1 nucleolar accumulation (Fig. 2A).

Only compounds affecting APE1 staining (17 out of 58 – Supplementary Table 1) were further screened through a proximity ligation assay (PLA) [22, 28, 29], which was designed to measure the extent of the residual APE1/NPM1 association upon cell treatment. As the AlphaScreen® was carried out using a recombinant GST-tagged form of APE1, we sought to exclude any possible artifact arising from the presence of the N-terminal tag by exploiting a HeLa cell line stably expressing a C-terminally FLAG-tagged version of APE1 [21]. The presence of the tag, in this case, was necessary to improve the quality of the PLA signal, and was already shown not to affect the interaction with NPM1 [22]. PLA was carried out by fixing cells immediately after treatment with the putative inhibitors and revealing the molecular association between APE1 and NPM1 with an anti-FLAG/anti-NPM1 pair of

antibodies (Fig. 2B). Quantification of the PLA signal allowed us to determine whether a particular compound was able to decrease the extent of the APE1/NPM1 interaction below that of vehicle-treated cells. This approach allowed us to narrow the number of putative inhibitors to eight molecules effectively able to interfere with the APE1/NPM1 interaction in cells (Fig. 2C – *top panel*). As anticipated by the immuno-fluorescence preliminary screening, some of the compounds that caused relocalization of APE1 (e.g. fiduxosin, spiclomazine) led to an accumulation of the interaction signal from the nucleoplasm to the cytoplasm (Fig. 2C – *bottom panel*). Treatment with other compounds, while leading to APE1 relocalization to the cytoplasm, did not produce any increase in the APE1/NPM1 interaction in this cellular compartment (e.g. SB 206553). In summary, our approach, exploiting a panel of secondary cell-based screens, allowed us to validate a set of eight low molecular weight compounds (i.e. FSCPX, troglitazone, SB 206553, ZM 241385, rotenone, spiclomazine, fiduxosin and myoseverin B) able to effectively displace the APE1/NPM1 interaction upon cell treatment. Rotenone, a respiratory chain poison and myoseverin B, known to bind microtubules [38], were excluded from additional analyses, because of their previously noted off-target effects.

To better characterize the six remaining hits, we analyzed their ability to interfere with the APE1 redox function. We exploited the JHH6 hepatocarcinoma cell line, which has already been shown to activate NF- κ B in an APE1-mediated and redox-dependent manner after a challenge with TNF- α [27]. JHH6 cells were transfected with a reporter plasmid bearing an interleukin 8 (IL-8) promoter with NF- κ B consensus sequences, and treated with the APE1/NPM1 inhibitors prior to TNF- α addition (Fig. 3A). Most of the tested molecules had no or little effect on the basal IL-8 promoter transcription; the effect of the APE1/NPM1 inhibitors on the redox-dependent promoter activation ranged from strong (e.g. troglitazone), mild (e.g. spiclomazine) to null (e.g. SB 206553). As expected [27], cell pre-treatment with the APE1 redox inhibitor E3330, strongly impaired the TNF- α -induced NF- κ B activation. Notably, the APE1/NPM1 inhibitor troglitazone had an effect similar to E3330, even at a tenfold lower concentration.

Selected compounds sensitize tumor cells to genotoxins and display anti-proliferative activity as single agent

We next focused on the anti-tumor properties of the six selected APE1/NPM1 inhibitors. We investigated their ability to sensitize HeLa cells to different genotoxins. Cell viability was measured through the MTS assay upon pre-treatment with the inhibitors, followed by challenge with methyl-methanesulphonate (MMS), which induces DNA alkylation that is repaired by the BER pathway [39]. Pre-treatment of cells with SB 206553 or spiclomazine resulted in a synergistic cytotoxicity when combined with MMS (Fig. 3B and Fig. 6). Likewise, through both cell counting and colony formation experiments, a sensitization effect was observed when combining fiduxosin, spiclomazine, or SB 206553 with the radiomimetic drug bleomycin, which is known to induce DNA strand breaks, along with a subset of oxidative DNA lesions that are repaired with the intervention of APE1 [40] (Fig. 3C – D and Fig. 6). Neither FSCPX nor ZM 241386 had a statistically significant effect on cell viability in combination with any genotoxic drug (not shown), and were therefore excluded from further characterization.

In addition to the enhancement of the cytotoxic effect of therapeutically relevant DNA damaging drugs, a desirable feature for an adjuvant to conventional anti-tumor therapy is an anti-proliferative activity. We tested whether the three inhibitors that promoted sensitization to MMS or bleomycin had any effect on cellular proliferation as a single agent. Remarkably, treatment of HeLa cells with either fiduxosin or spiclomazine, but not with SB 206553, resulted in a dose-dependent reduction of the cellular growth rate. This effect was visible at sub-lethal dosage (5 – 20 μ M), both upon acute (24 – 72 hours) or chronic (10 days) treatment (Fig. 4A – B and Fig. 6).

Characterization of the molecular target of the top hit compounds

In order to understand whether the molecular target of the inhibitors is APE1 or NPM1, we analyzed their binding properties using surface plasmon resonance (SPR). Despite the absence of any synergistic effect in combination with genotoxins (not shown), troglitazone was also characterized in these assays, given its powerful inhibitory effect on the APE1 redox activity (Fig. 3A). SPR experiments showed that the four most promising small molecules preferentially bind immobilized recombinant APE1, rather than NPM1 (Fig. 5A). Note that fiduxosin and spiclomazine could not be used at higher concentration due to their limited solubility in aqueous buffer. We then compared the binding affinity of SB 206553 and troglitazone to mutant forms of APE1. The interaction with both small molecules was severely impaired by the loss of the APE1 unstructured N-terminal extension, which is responsible for the interaction with NPM1 [21] (Fig. 5B, compare APE1 WT and the APE1 N 33 truncation mutant). Comparison of the APE1 WT and the APE1 KA form, which preserves the N-terminal extension but loses its ability to interact with NPM1 [21], revealed no difference in the binding capacity of the inhibitors (Fig. 5B). Taken together, these data suggest that APE1, rather than NPM1, is the preferred binding partner for some of the molecules active in this assay.

The above observations were further corroborated by *in vitro* assays assessing the endonuclease activity of APE1. These experiments showed that the inhibitors, at least slightly, inhibited the catalytic incision activity of purified recombinant APE1, with some molecules (i.e. spiclomazine and fiduxosin) having an effect comparable to that of the known APE1 inhibitor compound #3 [37] (Fig. 5C and Supplementary Fig. S1).

The APE1/NPM1 inhibitors do not impair ribosome biogenesis and only fiduxosin leads to accumulation of AP-sites

It is conceivable that the effects of the APE1/NPM1 inhibitors are mediated by the downregulation of one or both proteins. Notably, none of the three molecules effective in the combination studies (i.e. fiduxosin, spiclomazine and SB 206553) led to a significant reduction of either APE1 or NPM1 protein amounts (Fig. 7). Thus, neither the observed APE1/NPM1 interaction impairment, nor the measured effect on cell growth or sensitivity to DNA damage, can be ascribed to variations in the expression levels of these proteins.

Focusing on fiduxosin or spiclomazine, immuno-fluorescence analyses revealed that NPM1 maintains its nucleolar residence over a 72 hour treatment with either compound (Fig. 8A). Over the same time course, APE1 did not change its nucleolar localization when cells were

treated with spiclomazine. On the other hand, in cells treated with fiduxosin, APE1 was depleted from nucleoli during the first 48 hours (Fig. 8B), as already observed during the immuno-fluorescence based secondary screening (Supplementary Table 1). These observations suggest that the cytostatic effect of these two APE1/NPM1 inhibitors is not driven by the disruption of nucleolar integrity, as NPM1 localization was unaffected [31]. Fiduxosin, in this case, represented a notable exception, showing intact nucleoli, but relocation of APE1. In accordance with the absence of nucleolar disruption, the ribosome processing kinetics in HeLa cells treated with either fiduxosin or spiclomazine did not show any obvious alteration (Fig. 9A), indicating that other mechanisms might account for the impairment in cell proliferation.

To gain further insight into the mechanism of action of the selected APE1/NPM1 inhibitors we assessed any inhibitor-dependent accumulation of DNA damage through measurement of AP-sites and the use of the alkaline comet assay. Our data indicate that treatment of HeLa cells with fiduxosin induced a small, albeit significant, increase in the genomic AP-site content (Fig. 9B); spiclomazine or SB 206553, conversely, did not induce any major accumulation of DNA damage (Fig. 9B – C).

The cytotoxic effect of the APE1/NPM1 is common to different tumor cell lines

In order to extend our findings to other cell lines, we estimated the IC₅₀ of fiduxosin, spiclomazine and SB 206553 on a panel of tumor cell lines representing different cancer histotypes, such as hepatic (Huh7, JHH6), breast (HCC70, MCF7), glial (SF767) and ovarian (Ovcar5, TOV-112D). As shown in Table 1 and in Fig. 10, the IC₅₀ of the APE1/NPM1 inhibitors was similar among the samples, with few cell line-specific exceptions. Notably, HeLa cells were quite resistant to the cytotoxicity induced by the APE1/NPM1 inhibitors, having very high IC₅₀ values. Sensitization assays were also carried out on TOV-112D. Here, the sensitization effect of fiduxosin and spiclomazine was reproduced in the TOV-112D ovarian cancer cell line with bleomycin (Fig. 11A), but not with MMS. In addition, viability assays comparing the AML cell lines OCI/AML-2 (expressing wild-type NPM1) and OCI/AML-3 (expressing the NPM1c mutant and having an impaired APE1/NPM1 interaction [22]), showed a sensitizing effect for the SB 206553-bleomycin combination only in the cell line expressing wild-type NPM1 (Figure 11B).

Altogether, these data suggest that the cytotoxic and the sensitizing effects of spiclomazine, SB 206553 and fiduxosin are not restricted to HeLa cells. Moreover, these data further suggest that a functional APE1/NPM1 interaction is likely needed for the SB 206553 sensitization effect to occur in AML cell lines.

Discussion

The study presented herein represents the first attempt to target the APE1 protein in tumor cells through the disruption of its interactome. Exploiting the AlphaScreen® technology using full length recombinant proteins, we were able to screen a set of commercially available small molecule libraries for putative inhibitors of the APE1/NPM1 interaction. Although our data do not allow us to exclude the possibility of *in vivo* off target effects of the top-hits, using our proof of concept approach, we have successfully demonstrated that

structurally unrelated bioactive molecules are able to impair the APE1/NPM1 interaction in living cells. Moreover, the inhibition of this interaction leads to interesting drug potentiation and growth impairment phenotypes in tumor cell lines.

Targeting protein-protein interactions with small molecules is a challenging task. The issue is often complicated by the presence of large surface areas involved in the protein-protein binding, and by the lack of obvious binding pockets for the small molecules at many interaction interfaces [41, 42]. Since the APE1/NPM1 protein-protein interaction is known to involve the N-terminal region of both proteins [16, 17], we speculate that the compounds identified herein are targeting either the NPM1 oligomerization domain, or the APE1 unstructured extension [18], or even the APE1/NPM1 interface. The propensity of many positive hits to induce relocalization of APE1 implies that this class of inhibitors has greater affinity for the N-terminal region of the endonuclease, which is responsible for its nuclear localization [43] and interaction with NPM1 and rRNA [16–18]. The negative effects measured on APE1 redox function and on the endonuclease activity of the enzyme, together with the SPR data, strongly support preferential binding to APE1. In this study we did not carry out structural analyses to pinpoint the precise binding site of the hit compounds on APE1. Therefore, we cannot exclude that the small molecules may interact with different sites and/or affinities at the interaction interface. A preliminary observation of the molecular structure of the top hit compounds (Figure 6) suggests, for instance, that different chemical features of the molecules may affect their activity towards APE1. The experiment described in Figure 5C shows that both spiclomazine and fiduxosin inhibit APE1 with potency comparable to that of compound #3 [37]. Structure-activity relationship (SAR) studies have also shown that thiazolinic and thienopyridinic groups within compound #3 are associated with increased inhibitory activity of the molecule [37]. Interestingly, both fiduxosin and spiclomazine contain a thienopyrimidinic and thiazin moieties, presumably involved in the APE1 inhibition mechanism. The lower inhibitory effect of troglitazone and SB 206553 might be explained by (1) the presence of a less efficient thiazolinic moiety and (2) the absence of any sulfur-containing group. These features could explain the different behavior shown by molecules selected to target the same protein-protein interaction. However, these speculations need to be further addressed by targeted SAR investigations.

The impairment of the APE1 redox function might be, *per se*, an appealing feature for anti-cancer treatment, as already suggested [15], and a subset of the APE1/NPM1 inhibitors counteract the TNF- α -induced NF- κ B activation in hepatocarcinoma cells. Among the positive hits in this study we found troglitazone, a well-known anti-diabetic PPAR γ agonist that has been withdrawn from the market for its hepatotoxicity [44]. Notably, troglitazone has been proposed to exert anti-inflammatory action through the NF- κ B pathway [45], and various reports have described interesting anti-tumor properties of this compound in different cell models [46, 47]. These findings might be explained by our observation that the compound is a very potent APE1 redox inhibitor.

Polischouk and colleagues previously reported an interesting enhancing effect of the antipsychotic trifluoperazine (TFP) on bleomycin-mediated cytotoxicity [48]. One of the present APE1/NPM1 inhibitors, namely spiclomazine, is a member of the same class of drugs (i.e. phenothiazines), and we consistently observe here a synergic effect of bleomycin

and spiclomazine. Although TFP has been suggested to impair the non-homologous end joining process [48], an involvement of either APE1 [40] or NPM1 [19] in the elimination of bleomycin-induced DNA damage should not be excluded. Interestingly, spiclomazine has recently been proposed as a selective molecule for targeting pancreatic carcinoma, being able to reduce proliferation of cancer cell lines, with minor effects on non-transformed cell models. In the same study, spiclomazine was proposed to regulate the expression levels of apoptotic proteins, reducing the mitochondrial membrane potential, elevating reactive oxygen species levels and suppressing the metastatic potential of pancreatic carcinoma cell lines [49]. The redox activity of APE1, moreover, has been shown to be an ideal key target in pancreatic cancer [15]. In light of our findings, we speculate that the dual effect of spiclomazine on both the APE1 endonuclease and its redox activity might be linked to the effects observed on pancreatic tumor cell lines.

In this study, we show that molecules already known for different biological activities display previously uncharacterized anti-tumor properties. The sensitization effects observed when disrupting the APE1/NPM1 interaction, in combination with MMS or bleomycin, appear to be only partially related to a direct impact of the compounds on the BER capacity. In fact, while the inhibitory effect displayed by some molecules toward the APE1 endonuclease and redox activities is correlated with a negative impact on cellular proliferation (compare for instance fiduxosin and spiclomazine with SB206553), our data did not record any significant DNA damage accumulation under the experimental conditions tested (with fiduxosin being a clear exception). This apparent contradiction could reflect the low sensitivity of the assays used, since the negative effect on the APE1 endonuclease activity is fairly limited in terms of magnitude, at least at the concentrations tested *in vitro*. On the other hand, it is conceivable that non-canonical APE1 activities, distinct from its DNA repair-related function (e.g. RNA binding/cleavage, exonuclease, or RNase [6]), are involved in the response to the DNA damaging agents used in our experiments. Notably, genotoxins tested within this study are likely to induce the accumulation of RNA damage, along with DNA damage [50–52]. These considerations reflect the possible relevance of the APE1/NPM1 interaction in pathways different from DNA repair. Accordingly, the observed anti-proliferative effect of some of the inhibitors (i.e. spiclomazine and fiduxosin) may reflect a potential role of the APE1/NPM1 association in tumor cell proliferation, but not specifically in DNA repair, as previously suggested [21]. The impairment of any potential RNA cleansing function of the APE1/NPM1 complex [17] without obvious accumulation of DNA damage might explain why these inhibitors are cytostatic and cytotoxic. In addition, it is worth highlighting that the APE1/NPM1 inhibitors were identified for their ability to induce relocalization of APE1; therefore, it is possible that some of the effects observed (including the reduction in redox activity of APE1) are provoked by a relocalization of APE1 itself.

A limitation of this study is represented by the fact that our data cannot completely rule out that the effect of the small molecules is mediated in part by the disruption of the interaction between APE1 and other protein partners. It is clear, from our SPR data, that the N-terminal extension of APE1 is required for the interaction with two of the inhibitors. However, further interaction experiments exploiting the APE1 N 33 and the APE1 KA mutants to address the specificity of the inhibitors are not feasible. The results of the experiments, in fact, would be

biased in that both the mutant proteins display increased AP-endonuclease activity, impaired RNA binding and reduced binding activity toward known APE1 binding partners, as already reported [16, 17, 21]. To the best of our knowledge, however, our study represents the first example of the selection of low molecular weight protein-protein inhibitors targeting the flexible N-terminal region of APE1. It is worth underlining that the compounds identified in this study were screened using commercially available small molecule libraries, in the absence of any structure-based design. Further SAR studies will have to be carried out in order to improve the selectivity and specificity of the mechanism of action of these molecules.

In conclusion, these results show that bioactive molecules selected for their ability to impair the APE1/NPM1 association within cells are able to synergize with therapeutically relevant DNA damaging agents, increasing their cytotoxicity. Moreover, some of the molecules show interesting anti-proliferative activity as single agents. The anti-cancer properties of these molecules and their mechanism of action deserve further characterization; additional studies aimed at the improvement of the APE1/NPM1 inhibitors as novel therapeutic compounds are warranted. Design of more potent inhibitors might be useful for a thorough characterization of the relevance of this protein-protein interaction *in vivo*, as well as to improve existing therapeutic approaches in combination therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APE1	apurinic apyrimidinic endonuclease 1
BER	base excision repair
E3330	(2E)-3-[5-(2,3-dimethoxy-6-methyl 1,4-benzoquinoyl)]-2-nonyl-2-propenoic acid
HTS	high-throughput screening
NPM1	nucleophosmin
PLA	proximity ligation assay
MMS	methyl-methanesulphonate

MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
SPR	surface plasmon resonance
TNF-α	tumor necrosis factor- α

References

1. Kelley, MR. DNA repair in cancer therapy. Molecular targets and clinical applications. Academia Press; 2012. p. 341
2. Hellday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. *Nature Reviews*. 2008; 8:193–204.
3. Illuzzi JL, Wilson DM III. Base Excision Repair: Contribution to Tumorigenesis and Target in Anticancer Treatment Paradigms. *Curr Med Chem*. 2012; 19:3922–3936. [PubMed: 22788768]
4. Xanthoudakis S, Smeyne RJ, Wallace JD, Curran T. The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. *Proc Natl Acad Sci USA*. 1996; 93:8919–8923. [PubMed: 8799128]
5. Ludwig DL, MacInnes MA, Takiguchi Y, et al. A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity. *Mutat Res*. 1998; 409:17–29. [PubMed: 9806499]
6. Tell G, Fantini D, Quadrioglio F. Understanding different functions of mammalian AP endonuclease (APE1) as a promising tool for cancer treatment. *Cell Mol Life Sci*. 2010; 67:3589–3608. [PubMed: 20706766]
7. Sheng Q, Zhang Y, Wang R, et al. Prognostic significance of APE1 cytoplasmic localization in human epithelial ovarian cancer. *Med Oncol*. 2011; 29:1265–1271. [PubMed: 21479902]
8. Poletto M, Di Loreto C, Marasco D, et al. Acetylation on critical lysine residues of Apurinic/apyrimidinic endonuclease 1 (APE1) in triple negative breast cancers. *Biochem Biophys Res Commun*. 2012; 424:34–39. [PubMed: 22713458]
9. Di Maso V, Avellini C, Crocè LS, et al. Subcellular Localization of APE1/Ref-1 in Human Hepatocellular Carcinoma: Possible Prognostic Significance. *Mol Med*. 2007; 13:89–96. [PubMed: 17515960]
10. Tell G, Damante G, Caldwell D, Kelley MR. The Intracellular Localization of APE1/Ref-1: More than a Passive Phenomenon? *Antioxid Redox Signal*. 2005; 7:367–384. [PubMed: 15706084]
11. Robertson KA, Bullock HA, Xu Y, et al. Altered Expression of Ape1/ref-1 in Germ Cell Tumors and Overexpression in NT2 Cells Confers Resistance to Bleomycin and Radiation. *Cancer Res*. 2001; 61:2220–2225. [PubMed: 11280790]
12. Kakolyris S, Kaklamanis L, Engels K, et al. Human AP endonuclease I (HAPI) protein expression in breast cancer correlates with lymph node status and angiogenesis. *Br J Cancer*. 1998; 77:1169–1173. [PubMed: 9569057]
13. Madhusudan S, Smart F, Shrimpton P, et al. Isolation of a small molecule inhibitor of DNA base excision repair. *Nucleic Acids Res*. 2005; 33:4711–4724. [PubMed: 16113242]
14. Wilson DM III, Simeonov A. Small molecule inhibitors of DNA repair nuclease activities of APE1. *Cell Mol Life Sci*. 2010; 67:3621–3631. [PubMed: 20809131]
15. Kelley MR, Georgiadis MM, Fishel ML. APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1. *Curr Mol Pharmacol*. 2012; 5:36–53. [PubMed: 22122463]
16. Fantini D, Vascotto C, Marasco D, et al. Critical lysine residues within the overlooked N-terminal domain of human APE1 regulate its biological functions. *Nucleic Acids Res*. 2010; 38:8239–8256. [PubMed: 20699270]
17. Vascotto C, Fantini D, Romanello M, et al. APE1/Ref-1 Interacts with NPM1 within Nucleoli and Plays a Role in the rRNA Quality Control Process. *Mol Cell Biol*. 2009; 29:1834–1854. [PubMed: 19188445]

18. Poletto M, Vascotto C, Scognamiglio PL, Lirussi L, Marasco D, Tell G. Role of the unstructured N-terminal domain of the human Apurinic/Apyrimidinic Endonuclease 1 (hAPE1) in the modulation of its interaction with nucleic acids and Nucleophosmin (NPM1). *Biochem J.* 2013; 452:545–557. [PubMed: 23544830]
19. Colombo E, Alcalay M, Pelicci PG. Nucleophosmin and its complex network: a possible therapeutic target in hematological diseases. *Oncogene.* 2011; 30:2595–2609. [PubMed: 21278791]
20. Vassiliou GS, Cooper JL, Rad R, et al. Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. *Nat Genet.* 2011; 43:470–475. [PubMed: 21441929]
21. Lirussi L, Antoniali G, Vascotto C, et al. Nucleolar accumulation of APE1 depends on charged lysine residues that undergo acetylation upon genotoxic stress and modulate its BER activity in cells. *Mol Biol Cell.* 2012; 23:4079–4096. [PubMed: 22918947]
22. Vascotto C, Lirussi L, Poletto M, et al. Functional regulation of the Apurinic/apyrimidinic endonuclease APE1 by Nucleophosmin (NPM1): Impact on tumor biology. *Oncogene.* 2014; 33:2876–2887. [PubMed: 23831574]
23. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/ mutated nucleophosmin (NPMc + AML): biologic and clinical features. *Blood.* 2007; 109:874–885. [PubMed: 17008539]
24. Yun JP, Miao J, Chen GG, et al. Increased expression of nucleophosmin/B23 in hepatocellular carcinoma and correlation with clinicopathological parameters. *Br J Cancer.* 2007; 96:477–484. [PubMed: 17245342]
25. Londero AP, Tell G, Marzinotto S, et al. Expression and prognostic significance of APE1/Ref1 and NPM1 proteins in high-grade ovarian serous cancer. *Am J Clin Pathol.* 2014; 141:404–414. [PubMed: 24515769]
26. Yasgar A, Shinn P, Jadhav A, et al. Compound Management for Quantitative High-Throughput Screening. *JALA Charlottesv Va.* 2008; 13:79–89. [PubMed: 18496600]
27. Cesaratto L, Codarin E, Vascotto C, et al. Specific Inhibition of the Redox Activity of Ape1/Ref-1 by E3330 Blocks Tnf- α -Induced Activation of IL-8 Production in Liver Cancer Cell Lines. *PLoS One.* 2013; 8.10.1371/annotation/a610ee5c-525c-4d3c-b6b7-a4cde7b8db54
28. Gustafsdottir SM, Wennström S, Fredriksson S, et al. Use of proximity ligation to screen for inhibitors of interactions between vascular endothelial growth factor A and its receptors. *Clin Chem.* 2008; 54:1218–1225. [PubMed: 18499900]
29. Leuchowius KJ, Jarvius M, Wickström M, et al. High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol Cell Proteomics.* 2010; 9:178–183. [PubMed: 19864249]
30. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988; 175:184–191. [PubMed: 3345800]
31. Burger K, Mühl B, Harasim T, et al. Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J Biol Chem.* 2010; 16:12416–12425. [PubMed: 20159984]
32. Yamamori T, DeRicco J, Naqvi A, et al. SIRT1 deacetylates APE1 and regulates cellular base excision repair. *Nucleic Acids Res.* 2010; 38:832–845. [PubMed: 19934257]
33. Gros L, Ishchenko AA, Ide H, et al. The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway. *Nucleic Acids Res.* 2004; 32:73–81. [PubMed: 14704345]
34. Vascotto C, Cesaratto L, Zeef LA, et al. Genome-wide analysis and proteomic studies reveal APE1/Ref-1 multifunctional role in mammalian cells. *Proteomics.* 2009; 9:1058–1074. [PubMed: 19180539]
35. Meisenberg C, Tait PS, Dianova II, et al. Ubiquitin ligase UBR3 regulates cellular levels of the essential DNA repair protein APE1 and is required for genome stability. *Nucleic Acids Res.* 2012; 40:701–711. [PubMed: 21933813]
36. Simeonov A, Kulkarni A, Dorjsuren D, et al. Identification and characterization of inhibitors of human apurinic/apyrimidinic endonuclease APE1. *PLoS One.* 2009; 4:e5740.10.1371/journal.pone.0005740 [PubMed: 19484131]

37. Rai G, Vyjayanti VN, Dorjsuren D, et al. Synthesis, biological evaluation, and structure-activity relationships of a novel class of apurinic/aprimidinic endonuclease 1 inhibitors. *J Med Chem.* 2012; 55:3101–3112. [PubMed: 22455312]
38. Rosania GR, Chang YT, Perez O, et al. Myoseverin, a microtubule-binding molecule with novel cellular effects. *Nat Biotechnol.* 2000; 18:304–308. [PubMed: 10700146]
39. Kaina B, Ochs K, Grösch S, et al. BER, MGMT, and MMR in defense against alkylation-induced genotoxicity and apoptosis. *Prog Nucleic Acid Res Mol Biol.* 2001; 68:41–54. [PubMed: 11554312]
40. Fung H, Demple B. Distinct roles of Ape1 protein in the repair of DNA damage induced by ionizing radiation or bleomycin. *J Biol Chem.* 2011; 286:4968–4977. [PubMed: 21081487]
41. Jin L, Wang W, Fang G. Targeting protein-protein interaction by small molecules. *Annu Rev Pharmacol Toxicol.* 2014; 54:435–456. [PubMed: 24160698]
42. Arkin MR, Tang Y, Wells JA. Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. *Chem Biol.* 2014; 21:1102–1114. [PubMed: 25237857]
43. Jackson EB, Theriot CA, Chattopadhyay R, Mitra S, Izumi T. Analysis of nuclear transport signals in the human apurinic/aprimidinic endonuclease (APE1/Ref1). *Nucleic Acids Res.* 2005; 33:3303–3312. [PubMed: 15942031]
44. Rachek LI, Yuzefovych LV, Ledoux SP, Julie NL, Wilson GL. Troglitazone, but not rosiglitazone, damages mitochondrial DNA and induces mitochondrial dysfunction and cell death in human hepatocytes. *Toxicol Appl Pharmacol.* 2009; 240:348–354. [PubMed: 19632256]
45. Aljada A, Garg R, Ghanim H, et al. Nuclear factor-kappaB suppressive and inhibitor-kappaB stimulatory effects of troglitazone in obese patients with type 2 diabetes: evidence of an antiinflammatory action? *J Clin Endocrinol Metab.* 2001; 86:3250–3256. [PubMed: 11443197]
46. Yoshizawa K, Cioca DP, Kawa S, Tanaka E, Kiyosawa K. Peroxisome proliferator-activated receptor gamma ligand troglitazone induces cell cycle arrest and apoptosis of hepatocellular carcinoma cell lines. *Cancer.* 2002; 95:2243–2251. [PubMed: 12412180]
47. Okura T, Nakamura M, Takata Y, Watanabe S, Kitami Y, Hiwada K. Troglitazone induces apoptosis via the p53 and Gadd45 pathway in vascular smooth muscle cells. *Eur J Pharmacol.* 2000; 407:227–235. [PubMed: 11068018]
48. Polischouk AG, Holgersson A, Zong D, et al. The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non-small cell lung carcinoma cells to DNA double-strand break-induced cell death. *Mol Cancer Ther.* 2007; 6:2303–2309. [PubMed: 17699725]
49. Zhao W, Li D, Liu Z, Zheng X, Wang J, Wang E. Spiclomazine induces apoptosis associated with the suppression of cell viability, migration and invasion in pancreatic carcinoma cells. *PLoS One.* 2013; 8:e66362.10.1371/journal.pone.0066362 [PubMed: 23840452]
50. Vågbø CB, Svaasand EK, Aas PA, et al. Methylation damage to RNA induced in vivo in *Escherichia coli* is repaired by endogenous AlkB as part of the adaptive response. *DNA Repair (Amst).* 2013; 12:188–195. [PubMed: 23276627]
51. Magliozzo RS, Peisach J, Ciriolo MR. Transfer RNA is cleaved by activated bleomycin. *Mol Pharmacol.* 1989; 35:428–432. [PubMed: 2468077]
52. Carter BJ, De Vroom E, Long EC, Van Der Marel GA, Van Boom JH, Hecht SM. Site-specific cleavage of RNA by Fe(II)bleomycin. *Proc Natl Acad Sci USA.* 1990; 87:9373–9377. (45). [PubMed: 1701259]

Fig. 1. Development of an HTS assay to individuate APE1/NPM1 interaction inhibitors
(A) Schematic representation of the HTS assay principle. The interaction between full length GST-APE1 and His₍₆₎-NPM1 is monitored in solution upon excitation at 680 nm. The glutathione-coated donor beads release singlet oxygen, which excites only proximal (200 nm) Ni²⁺-chelating acceptor beads. The presence of APE1/NPM1 interaction is revealed by luminescence emission at 520–620 nm. **(B)** Graphic summary reporting the Z' screening score; a stable value above 0.65 was maintained throughout the screening. **(C)** Representative curves observed from eight screening hits selected during downstream analyses. The graph reports the concentration-dependent loss of APE1/NPM1 interaction signal for the indicated compounds, relative to the vehicle-treated samples. Structures and additional data associated with the best characterized hits are presented in Figure 6.

Fig. 2. Secondary orthogonal assays to validate the hits from the primary screening

(A) Representative immuno-fluorescence analysis on HeLa cells treated with positive hits from the primary screening. The subcellular localization of APE1 was assessed through immuno-staining with an anti-APE1 antibody (red). Note the absence of nucleolar accumulation of APE1 upon stimulation with ZM 241385. Q LUT (quantitative color look-up table) rendering highlights the increased cytoplasmic localization after SB 206553 treatment. (B) PLA-based secondary validation of the APE1/NPM1 inhibitors. Representative PLA experiment performed on fixed HeLa cells upon treatment with fiduxosin (10 μ M, 16 hours) or troglitazone (10 μ M, 8 hours). APE1 expression is detected by using an anti-FLAG antibody (green), while PLA signal is visible as red dots. Control reaction is carried out without the anti-NPM1 antibody and shows no or little PLA signal. (C) The extent of APE1/NPM1 association upon treatment with a subset of inhibitors was measured through PLA, and the effect of the inhibitors on such interaction is reported as a boxplot graph (*top panel*). The average number of interaction spots scored in vehicle-treated cells was used as reference and arbitrarily set to 1; the relative extent of APE1/NPM1 association upon inhibition of the interaction was calculated by scoring the average of PLA signals in the inhibitor-treated cells. Cell challenge with the indicated molecules determines a statistically significant reduction in the extent of cellular APE1/NPM1 interaction. Boxplot

representation reporting the median cytoplasm-to-nucleus ratios of the amount of PLA interaction spots scored in HeLa cells upon treatment with the indicated APE1/NPM1 inhibitors (*bottom panel*). Black bars in the graphs report the median for each distribution. A red line indicates the median of the vehicle-treated cells, for easier identification of differences. The amount and the localization of the APE1/NPM1 interaction signals were assessed as described in “*Materials and Methods*”. N = 35, *: p<0.05, **: p<0.01, ***: p<0.001, NS: not statistically significant.

Fig. 3. Inhibition of the APE1/NPM1 interaction has differential impact on the APE1 redox activity and sensitizes HeLa cells to genotoxic treatment

(A) The APE1/NPM1 inhibitors differently affect the redox function of APE1. JHH6 cells were transfected with a luciferase reporter bearing the IL-8 promoter with NF- κ B consensus sequences [27] and then pre-treated with the indicated APE1/NPM1 inhibitors. After five hours cells were challenged with TNF- α and luciferase activity was measured to assess the APE1-mediated NF- κ B activation. While the inhibitors activity on APE1 basal redox activity was negligible, they showed differential inhibitory capacity over TNF- α -mediated NF- κ B activation. Results reported are the mean $\pm$ SD of at least three independent experiments, *: $p < 0.05$, **: $p < 0.01$. E3330 was used as positive control for inhibition of APE1. (B) SB 206553 and spiclomazine sensitize HeLa cells to MMS. HeLa cells were pre-treated with 10 μ M SB 206553 or spiclomazine for 8 hours and then co-treated for further 8 hours with unchanged inhibitor concentration and MMS. The effect of SB 206553 and spiclomazine as single agent was, respectively, $1.08 \pm 0.08\%$ and $0.99 \pm 0.04\%$ relative to the vehicle. (C) Bleomycin cytotoxicity is increased in presence of either SB 206553 or fiduxosin. HeLa cells were co-incubated with increasing amounts of bleomycin in presence of 10 μ M of the indicated APE1/NPM1 inhibitor for 1 hour. Cell viability was monitored 48 hours later through cell counting. The effect of SB 206553 and fiduxosin as single agent was, respectively, $1.22 \pm 0.02\%$ and $0.86 \pm 0.06\%$ relative to the vehicle. (D) Combination with spiclomazine increases the potency of bleomycin. HeLa cells were co-incubated with increasing amounts of bleomycin in presence of 10 μ M spiclomazine for 1 hour. Cell viability was monitored through colony formation assays, showing synergic behavior of bleomycin and spiclomazine. The cell killing effect of spiclomazine as single agent was $0.90 \pm 0.08\%$ relative to the vehicle. Data reported are the mean $\pm$ SD of at least three independent experiments, *: $p < 0.05$, **: $p < 0.01$.

Fig. 4. Fiduxosin and spiclomazine, but not SB 206553, impair cell growth as single agents (A) HeLa cells were grown in presence of the indicated APE1/NPM1 inhibitor (10 or 20 μM) and cell proliferation was assessed after 24, 48 and 72 hours through cell counting. Acute treatment with fiduxosin or spiclomazine, but not with SB 206553, affects the cellular proliferation rate in a dose-dependent fashion. (B) HeLa cells were chronically stimulated with increasing amounts of the indicated inhibitor during colonies growth. The percentage of surviving colonies relative to vehicle-treated cells is reported. Inhibition of the APE1/NPM1 interaction using fiduxosin or spiclomazine, but not SB 206553, results in a dose-dependent impairment of colony formation capacity. Data reported are the mean ± SD of at least three independent experiments, *: $p < 0.05$.

Fig. 5. The APE1/NPM1 inhibitors show preferential binding to APE1

(A) Histogram representation of the SPR analysis on the small molecules-protein interaction studies. The graph reports the RU_{max} for each binding experiment using the indicated concentration of analyte on immobilized recombinant APE1 or NPM1; Note the lower response for NPM1. E3330 was used as positive control for interaction with APE1. (B) The indicated recombinant APE1 forms were compared for the interaction with SB 206553 or troglitazone. The histogram reports the RU_{max} for each binding experiment using the indicated concentration of analyte; signal was normalized to the molecular weight of each small molecule. (C) The APE1/NPM1 inhibitors negatively impact on the AP-site incision activity of APE1. APE1 endonuclease activity was measured *in vitro* as indicated in the “Materials and Methods” section in presence of 100-fold molar excess of the indicated molecules. APE1 activity is reported as percentage of substrate converted to product as a function of time. The APE1 inhibitor compound #3 [37] was used as positive control; the APE1 AP-site incision profile in presence of this inhibitor is highlighted in red. Data are expressed as mean ± SD of three technical replicates from two independent assays.

Compound	Structure	Redox inhibition	AP-site incision inhibition	Sensitization to genotoxins		Anti-proliferative activity
				MMS	Bleomycin	
Troglitazone		++	+	-	NA	NA
Fiduxosin		+	++	-	+	+
SB 206553		-	+	+	+	-
Spiclomazine		+	++	+	+	+

Fig. 6. Sensitizing and anti-proliferative capacity of the APE1/NPM1 inhibitors

The figure schematically summarizes the properties for each top-hit inhibitor. For every compound analyzed the “+” symbol indicates the presence of sensitization in combination with the indicated genotoxin. “++” and “+” indicate the strength of the phenotype on the APE1 redox and AP-endonuclease functions, as strong or mild, respectively. The anti-proliferative activity of each molecule as single-agent is also reported, along with the chemical structure and the commercial name of the compound. NA: not available, “-”: no effect.

Fig. 7. Inhibition of the APE1/NPM1 interaction does not affect the expression levels of APE1 or NPM1

Representative Western blotting showing the APE1 and NPM1 expression levels in HeLa whole cell extracts. After treatment with the indicated inhibitors (10 μ M for 16 hours) no significant variation of the protein expression pattern is observed.

Fig. 8. NPM1 localization is not affected by prolonged treatment with fiduxosin or spiclomazine; whereas APE1 nucleolar accumulation is affected by fiduxosin only

(A) Representative immuno-fluorescence analysis on HeLa cells treated with either fiduxosin or spiclomazine (20 μM for the indicated time points). NPM1 staining (red) shows the constant nucleolar accumulation of the protein throughout the treatment. Merged panels show the superimposition of NPM1 and TO-PRO-3 staining (blue). (B) Representative micrographs showing a typical immuno-fluorescence analysis on HeLa cells treated with either fiduxosin or spiclomazine (20 μM for the indicated time points). APE1 staining (green) shows a decreased nucleolar accumulation of the protein in presence of fiduxosin, but not upon treatment with spiclomazine. Merged panels show the superimposition of APE1 and TO-PRO-3 staining (blue).

Fig. 9. Inhibition of the APE1/NPM1 interaction does not affect rRNA processing or DNA integrity

(A) rRNA maturation kinetics were monitored in HeLa cells as described in “*Materials and Methods*” after treatment with either spiclomazine or fiduxosin (20 μ M) for the indicated time. The proteasome inhibitor MG132 (50 μ M, 2 hours) was used as positive control [31]. Cell treatment under these experimental conditions does not lead to any obvious rRNA processing impairment. V: vehicle. (B) Genomic AP-site content quantification on HeLa cells upon treatment with the APE1/NPM1 inhibitors does not reveal any major damage accumulation. HeLa cells were treated with fiduxosin or spiclomazine (10 μ M, 48 hours), SB 206553 (20 μ M, 16 hours), or MMS (250 μ M, 16 hours) as positive control. AP-sites amount was calculated as described in “*Materials and Methods*”. Data are expressed as mean $\pm$ SD of three replicate measurements, *: $p < 0.01$. (C) Comet assay performed on HeLa cells shows the lack of accumulation of alkali-sensitive sites on genomic DNA after treatment with the APE1/NPM1 inhibitors (16 hours). HeLa cells were exposed to fiduxosin, spiclomazine (30 μ M), SB 206553 (100 μ M), or H₂O₂ (200 μ M, 10 minutes) as positive control, and tail moment was measured as described. The boxplot reports the median tail moment in at least 100 cells per condition. **: $p < 0.001$.

Fig. 10. IC₅₀ estimates for spiclomazine, fiduxosin and SB 206553 on different tumor cell lines
 Representative cytotoxicity curves used for the calculation of the IC₅₀ of selected APE1/
 NPM1 inhibitors. The indicated cell lines were grown in presence of increasing
 concentrations of spiclomazine (**A**), fiduxosin (**B**) or SB 206553 (**C**) for 48 hours; cell
 viability was measured through the MTS assay. Maximum dosage was limited by the poor
 solubility of fiduxosin and spiclomazine in aqueous medium. Values plotted are the average
 ± SD of five experimental replicates. 50% survival is highlighted by a red line.

Fig. 11. Spiclomazine and fiduxosin sensitize TOV-112D cells to bleomycin, while SB 206553 shows differential sensitization to bleomycin in AML cell lines

(A) TOV-112D ovarian cancer cells were incubated with 20 μM of either fiduxosin or spiclomazine for 7 hours and subsequently challenged with increasing amounts of bleomycin for 1 hour in presence of unchanged inhibitor concentration. Cell viability was monitored 48 hours later through cell counting. The cell killing effect of fiduxosin and spiclomazine as single agent was, respectively, $0.98 \pm 0.07\%$ and $0.91 \pm 0.10\%$ relative to the vehicle. Data reported are the mean $\pm$ SD of at least three independent experiments, *: $p < 0.05$, **: $p < 0.01$. (B) OCI/AML-2 or OCI/AML-3 cells were incubated for 24 hours with 20 μM SB 206553, challenged with bleomycin (1 hour, 100 μg/ml), washed and incubated with unchanged SB 206553 concentration for further 24 hours. Cell viability was assessed through cell counting. Data reported are the mean $\pm$ SD of at least four experimental replicates, **: $p < 0.01$. The effect of SB 206553 relative to the vehicle as single agent was $1.40 \pm 0.06\%$ for OCI/AML-2 and $1.27 \pm 0.09\%$ for OCI/AML-3 cells.

Table 1
IC₅₀ estimates for spiclomazine, fiduxosin and SB 206553 on different tumor cell lines.

Compound	HeLa	Huh7	HCC70	JHH6	SF767	MCF7	Ovcar5	TOV-112D
Fiduxosin	NA	39.8	43.5*	31.3	43.3*	NA	52.7*	45.6*
Spiclomazine	NA	38.0	46.3*	48.8*	NA	NA	NA	40.2
SB 206553	109.4*	NA	56.0	84.2	55.8	77.5	NA	NA

The indicated cell lines were exposed to the APE1/NPM1 inhibitors for 48 hours and cell viability was assessed using the MTS assay. IC₅₀ values are expressed in μ M and were calculated using the GraphPad Prism v6.0 software. Starred values (*) are slightly above the experimental curve and were therefore extrapolated using the same software. "NA" indicates an IC₅₀ far above the tested range.