Original Article

Mutant p53 as a therapeutic target for the treatment of triple-negative breast cancer: Preclinical investigation with the anti-p53 drug, PK11007

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A B S T R A C T

The identification of a targeted therapy for patients with triple-negative breast cancer (TNBC) is one of the most urgent needs in breast cancer therapeutics. The p53 gene is mutated in approximately 80% of patients with TNBC, and is a potential therapeutic target for patients with this form of breast cancer. The 2-sulfonylpyrimidine compound, PK11007, preferentially decreases viability in p53-compromised cancer cell lines. We investigated PK11007 as a potential new treatment for TNBC. IC50 values for inhibition of proliferation in a panel of 17 breast cell lines by PK11007 ranged from 2.3 to 42.2 μM. There were significantly lower IC50 values for TNBC than for non-TNBC cell lines (p = 0.03) and for p53-mutated cell lines compared with p53 WT cells (p = 0.003). Response to PK11007 however, was independent of the estrogen receptor (ER) or HER2 status of the cell lines. In addition to inhibiting cell proliferation, PK11007 induced apoptosis in p53 mutant cell lines. Using RNAseq and gene ontology analysis, we found that PK11007 altered the expression of genes enriched in pathways involved in regulated cell death, regulation of apoptosis, signal transduction, protein refolding and locomotion. The observations that PK11007 inhibited cell proliferation, induced apoptosis and altered genes involved in cell death are all consistent with the ability of PK11007 to reactivate mutant p53. Based on our data, we conclude that targeting mutant p53 with PK11007 is a potential approach for treating p53-mutated breast cancer, including the subgroup with TN disease.

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Introduction

p53 (TP53) is the most frequently mutated gene in several different human cancer types, including breast cancer [1–3]. In breast cancer, the frequency of mutation depends on the molecular subtype of the disease; mutant p53 being most prevalent in patients with the basal/triple negative (TN) subtype and least prevalent in those with luminal A tumors [4–8]. Thus, following whole exome sequencing on 510 invasive breast cancers, the Cancer Genome Atlas Network detected p53 mutations in 12% of samples from patients with luminal A type disease, 29% of samples in those with luminal B type, 72% in those with HER2-enriched type and in 80% in those with the basal subtype [5]. In this study, 80% of the 93 basal samples investigated were categorized as triple-negative (TN), i.e., negative for estrogen receptors (ER), progesterone receptors (PR) and HER2. The clinical consequence of the absence of these 3 biomarkers is that targeted therapy is currently unavailable
for TN patients [9,10]. At present, therefore, the identification and validation of a targeted therapy for TN breast cancer is an urgent requirement. As p53 is mutated in the vast majority of patients with TN breast cancer [4–8], it is a potential therapeutic target for women with this molecular form of the disease. But, until recently, targeting mutant p53 has proved to be difficult. p53 is inactivated by mutation in two principal ways: contact mutations that weaken the binding to p53 response elements; and structural mutations that lower the stability of the core domain [11]. The activity of structural mutants may be restored in some cases by small molecules that bind to and stabilise the core domain [11,12].

PRIMA-1 and its methylated derivative, PRIMA-1MET (APR-246) [13–21] alkylate cysteine residues in p53 and inhibit cancer cell growth in several different preclinical systems [13–21]. Indeed, APR-246 has undergone evaluation in a phase I clinical trial [22] and is currently undergoing further clinical evaluation in a phase IIb clinical trial in patients with high grade serous ovarian carcinoma [23] (NCT02089843).

Bauer et al. [24] have identified 5-chloro-2-methanesulfonic acid derivative, PK11007 as alkylating specific residues in p53. This modification resulted in raised protein melting temperature ($T_m$) of the DNA binding domain of the structural mutant p53-Y220C by 1.5 K at a concentration of 1 mM. The compound also covalently modified cysteines 182 and 277 without compromising its DNA-binding activity. Diverse substitutions on the 2-sulfonpyrimidine scaffold of PK11000 were subsequently tested, giving rise to PK11007 and PK11010 (Fig. 1, Suppl.). Both the 2-sulfonylpyrimidine scaffold of PK11000 were subsequently tested, giving rise to PK11007 and PK11010 (Fig. 1, Suppl.). Both the 2-sulfonylpyrimidine scaffold of PK11000 were subsequently tested, giving rise to PK11007 and PK11010 (Fig. 1, Suppl.). PK11007 preferentially inhibits viability of cancer cells expressing mutant p53 rather than wild-type (WT) p53 [23]. In addition to activation of mutant p53, PK11007 also induces cell death, possibly by depleting cellular GSH and ROS levels [24].

Here, we investigated the inhibitory potential of PK11000, PK11007 and PK11010 for cell growth in TN breast cancer using a panel of 17 breast cell lines.

Materials and methods

Cell culture

The following panel of breast cell lines were used; Hs578T(18), MDA-MD-468, HCC1937, HCC1143, BT20, BT474, (all TN), Sum 159, MDA-MD-453, SKBR3, BT474, JIMT1, UACC-812 (all HER2 amplified), T47D, ZR-75-1, MCF7 (all luminal), MCF12A and MCF10A (immortalised breast epithelial). All cell lines, apart from Hs578T(18) which was supplied by Dr Susan McDonell, University College, Dublin, were purchased from the American Type Culture Collection (ATCC) and maintained as previously described [19]. Cell line identity was confirmed by analysis of Short Term Repeat Loci. In addition, cells were routinely tested for mycoplasma infection.

Cell viability assay

Cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). To test the effect of PK11000, PK11007 and PK11010 on proliferation, cells were plated at a density of 1 × 10^4 cells/well in 96-well flat-bottomed plates (Sigma-Aldrich). Following overnight incubation, quadruplicate wells were treated with the above listed compounds at concentrations ranging from 0 to 50 μM. After 5 days, 0.5 mg/ml MTT was added to each well and incubated at 37 °C for 5 h. Media was aspirated, and 200 μl of DMSO was added to each well, for 5 min. Absorbance was measured at a wavelength of 550 nm on a microplate reader (Multiscan Ascent, Labsystems).

Detection of p53, p63 and p73

Extracted proteins (80 μg) were separated on a 12% polyacrylamide gel (Bioscience). Protein was transferred to a nitrocellulose membrane (Millipore) and blocked for 1 h at room temperature in 2% low-fat milk (Marvel instant dried skimmed milk) in Tris-buffered saline/0.1% Tween 20 (TBS). The membranes were stained overnight for p53, p63 and p73 as previously described [19]. Positivity was defined as the presence of a visible band at the location predicted from the relevant molecular mass of the individual proteins. In addition to Western blotting, p53 was also measured by ELISA (PathScan Total p53 Sandwich ELISA Antibody Pair, Cell Signalling Technology, #7844s). p53 knockdown

Cells were transiently transfected with pre designed ‘Flexiblute’ TP53 siRNA sequence (Qiagen). For HCC1143 cells, the HS_TP53_13 target sequence TGGTGAACCTTACAGGATCTAA was used; for MDA-MB-468 cells, the HS_TP53_3 target sequence AAGGAATTTTCTTGCCTGGAGT was used and for MDA-MB-231 cells, both the HS_TP53_7 target and HS_TP53_9 target sequences were used. The AllStars siRNA sequence (Qiagen) was used as the negative control. Briefly, siRNA (40 nM) was diluted in OptiMem to a final volume of 200 μl. Hiperfect (9 μl/200 μl) was added and the solution was vortexed for 10 s, prior to incubation for 15 min at room temperature. Five × 10^4 cells were seeded in a 24-well plate and RNA secondary complex was added in a drop-wise fashion onto the cells. Cells were incubated under normal growth conditions for 24 h. Complexes were replenished, and cells were incubated for a further 6 h. Cells were trypsinized, counted and seeded at a density of 2 × 10^3 on a 96 well plate. Following overnight incubation, cells were treated with 5 μM PK11007 or DMSO. After 48 h incubation, cell viability was assessed by the MTT assay and p53 levels by Western blotting, using the p53 DO-1 antibody, and GADPH as a loading control.

Immunofluorescent staining

SKBR3 cells (which contain the p53 structural mutation, Arg175His) were seeded to confluency in an 8 well chamber slide. The following day cell were treated with 25 μM or 50 μM PK11007 or DMSO control for 3 h. Cells were fixed with 1% glutaraldehyde for 20 min and blocked with PBS containing 10% goat serum (Cell Signalling) and 0.1% Triton-X for 1 h. The cells were stained overnight with anti-wildtype p53 antibody PAb1620 (Merck) (1:300), or anti-mutant p53 antibody PAb240 (Abcam) (1:500) at 4 °C. Cells were washed (×3) and incubated with goat anti-rat secondary antibody (Santa Cruz) (1:2000) for 1 h at room temperature. Dapi (Fisher Scientific) (1:2000) was used to stain cell nuclei. Slides were mounted with ProLongTM Gold anti-fade reagent (Biosciences) and stored at 4 °C. The following day, stained cells were visualised using a Leitz DM40 microscope (Leica Microsystems) and images were captured using the AxioCam system and AxioVision 3.0.6.

Quantification of wild-type and mutant p53 protein by flow cytometry

SKBR3 or MCF7 cells were seeded to confluency in a 24-well plate. The following day, cells were treated with 25 μM or 50 μM of drug or control for 3 h. Cells were fixed with 0.5% paraformaldehyde for 5 min. The cells were subsequently permeabilised and blocked with PBS containing 10% goat serum (Cell Signalling) and 0.1% Triton-X for 15 min. The cells were then stained overnight with anti-wildtype p53 antibody PAb1620 (Merck Millipore Ltd.) (1:500), or anti-mutant-p53 antibody PAb240 (Abcam) (1:500) at 4 °C and incubated with goat anti-rat secondary antibody (Santa Cruz) (1:2000) for 1 h at room temperature. FACS analysis was performed using a BD FACSComp™. Data was analysed using Flowjo software v10.1.3.

Aptoptosis assays

Cells were seeded in 6-well plates (Sigma-Aldrich) at a density of 2 × 10^5 cells/well. Following overnight incubation, wells were treated with varying concentrations of the compounds in low serum (2% FBS) supplemented RPMI 1640. Staining for annexin-V and propidium iodide was carried out using the Annexin V-FITC Apoptosis Detection Kit (eBioscience), according to the manufacturer’s instructions. FACS analysis was immediately performed using a BD FACSCalibur™. Data was analysed using BD CellQuest™ Pro, version 6.0 software.

RNA isolation and real-time PCR

RNA from cell lines was isolated using the RNeasy Mini kit (Qiagen). One μg of RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). All primers were purchased from Qiagen. GADPH was used as a loading control. The amplification process was carried out as recommended by Qiagen for the Roche LightCycler480.

RNA-seq analysis

HCC1143 cells were treated with 2.5 μM of PK11007 or control (DMSO) in triplicate for 12 h. RNA was extracted and purified using RNeasy Mini Kit (Qiagen) and the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer’s instructions. RNA integrity was determined using a Bioanalyzer (Agilent, Santa Clara, CA). Libraries were prepared for RNASeq using the Illumina TruSeq stranded mRNA protocol kit according to the manufacturer’s instructions (Illumina, San Diego, CA). Sequencing was performed using an Illumina HiSeq 2500, to produce 51bp single-end reads and quality control was conducted using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to human genome version 19, using the sequence aligner Subread [25] via the bioconductor R.
package Rsubread (https://bioconductor.org/packages/). The data was scale normalised using the TMM (trimmed mean of M values (TMM)) normalisation method [26]. Differential expression was determined using the ebayes function of the R package Limma [27]. An adjusted p-value of less than 0.05 and a fold change greater than 1.4-fold was considered significant. The p-values were adjusted using the Benjamini and Hochberg method [28]. The package Limma was chosen here for differential expression analysis as it was found to be particularly robust when dealing with small sample sizes [29]. Gene ontology (GO) analysis was performed using the R package goseq [30], a package specifically designed for performing GO analysis on RNAseq data so as to overcome the inherent bias in RNAseq data for over-detecting long and highly expressed transcripts. All calculations were carried out in the R statistical environment (https://cran.r-project.org/).

**Statistical analysis**

Data were analysed using Prism version 5.0b software (GraphPad Software). The IC₅₀ (concentration required to inhibit growth by 50%) for each compound and combination index (CI value) was determined using CalcuSyn software (Biosoft). For the experiments involving drug combinations, CI < 0.9 indicated synergism, 0.9—1.1 indicated an additive effect and CI > 1.1 indicated an absence of an enhanced effect from the combination [31]. IC₅₀ values were compared for the three drugs across the cell lines using repeated measures ANOVA. The correlation coefficients were calculated using Spearman’s non-parametric test. All means were compared using a Student’s t-test (paired or unpaired depending on the data). P values < 0.05 were considered significant.

**Results**

**Effects of PK11000, PK11007 and PK11010 on proliferation of breast cell lines**

Initially, the growth inhibitory effects of PK11000, PK11007 and PK11010 were investigated in a panel of 17 breast cell lines. Using the MTT assay, IC₅₀ values ranged from 2.3 to 42.2 µM for PK11007, from 2.5 to >50 µM for PK11000 and from 15.4 to >50 µM for PK11010 (Fig. 1A–C; Table 1, Suppl.). Of the 3 compounds, the lowest IC₅₀ values were found for PK11007 (p = 0.03) (Fig. 1D), i.e., PK11007 was found to be the most sensitive inhibitor of cell growth. Response to PK11010 correlated significantly with that of PK11000 (p = 0.04) and PK11007 (p = 0.01) (Fig. 1E and F). However, no correlation was seen between response to PK11000 and PK11007 (p = 0.38). Since, PK11007 was found to be the most potent inhibitor of the 3 compounds investigated, we focused on it for the rest of the study.

**Relationship between response to PK11007 and molecular subtypes of cell lines**

As TN breast cancer currently lacks a targeted therapy treatment option, our primary aim was to identify an inhibitor of cancer cell growth for this subgroup of patients. TN cell lines were significantly more responsive to PK11007 than non-TN cell lines (Fig. 2A), with significantly lower IC₅₀ values (p = 0.03). To understand further the greater sensitivity of the TN cell lines versus the non-TN cell lines, we compared p53 protein levels in the 2 groups of cell lines. p53 protein levels tended to be higher in the TN compared to the non-TN cell lines (p = 0.05) (Suppl. Fig. 2A). This finding suggests that higher levels of p53 protein in the TN cell lines compared to the non-TN cell lines may explain the greater sensitivity of the former to PK11007. In contrast, response to PK11007 was independent of the ER status and HER2 status of the cell lines (p = 0.65 and p = 0.16, respectively) (Suppl. Fig. 2 B).

**Relationship between response to PK11007 and cell line p53 mutational Status/p53 protein levels**

p53 mutated cell lines were significantly more sensitive to PK11007 than p53 WT cells (Fig. 2B, p = 0.003). However, response was independent of whether the mutation was contact or structural (p = 0.6) (Suppl. Fig. 2C). As the mutant p53 protein in malignant
cells is less susceptible to degradation than the WT protein, it accumulates in cancer cells [32]. Indeed, the accumulation of p53 protein in malignant cells has been widely used in the past as a surrogate marker for the presence of mutation. Accordingly, we next compared response to PK11007 with p53 protein levels. p53 protein was present in 9 cell lines but undetectable in 8 cell lines, using Western blotting. IC<sub>50</sub> values were significantly lower in cell lines expressing detectable p53 protein levels than those with an absence of p53 (p = 0.02) (Fig. 2C). Furthermore, IC<sub>50</sub> values for PK11007 correlated strongly and significantly inversely with p53 concentrations quantified by ELISA (p = 0.01, r = 0.59, n = 17) (Fig. 2D). In contrast, there was not a significant relationship between response to PK11007 and levels of the two p53 family members, p63 or p73 (p = 0.06 and p = 0.43, respectfully) (Suppl. Fig. 2D and E).

**Effect of p53 knockdown on response to PK11007**

To establish if PK11007 was inhibiting cell growth via p53, we decreased expression of the mutant protein using 3 independent siRNA sequences. Following transient transfection, levels of p53 protein were found to be significantly decreased in the 3 cell lines investigated (for HCC1143 cells, mean % decrease was 79.3% using siRNA_13; for MDA-MD-231 cells, mean % decrease was 79.2% using siRNA_7 and 73.6% using siRNA_9 and for MDA-MB-468 cells, the mean % decrease was 88.6% using siRNA_9). This reduction in p53 protein levels resulted in a significant decrease in the growth inhibitory effects of PK11007, in all 3 cell lines investigated (Fig. 2E). These findings are consistent with p53 being a target for PK11007.

**Effect of PK11007 on induction of apoptosis**

As induction of apoptosis is one of the best-known functions of WT p53, we investigated the effects of PK11007 on this process. PK11007 was found to induce apoptosis in both a time and concentration-dependent manner in the two p53-mutated TNBC cell lines investigated, HCC1143 and MDA-MB-468 (Fig. 3A and B), but not in the WT-p53 cell line, MCF-7 (Fig. 3C) (Suppl. Fig. 3). Consistent with its ability to induce apoptosis, treatment with PK11007 upregulated expression of the p53 WT canonical genes, PUMA and NOXA in a concentration-dependent manner in the two p53-mutated TNBC cell lines investigated, i.e., in HCC1143 and MDA-MB-468 cells. Although not implicated in apoptosis but in cell cycle arrest, addition of PK11007 also upregulated p21 expression in a time and concentration-dependent manner (Fig. 3D).

**Effect of PK11007 on global gene expression**

To investigate more global effects of PK11007, we treated HCC1143 cells with the compound and analysed differentially expressed genes using RNA-seq analysis. In total, 390 genes were significantly upregulated and 234 genes downregulated following treatment. Table 2 (Suppl.) lists the top 20 most upregulated and downregulated genes, by fold change. GO terms significantly enriched in the differentially regulated genes are listed in Table 1. As might be expected from targeting p53, the list includes pathways implicated in regulated cell death, regulation of apoptosis, signal transduction, protein refolding and locomotion.

Next, we compared our differentially regulated gene lists from PK11007 treated cells with those from another study which used...
Fig. 3. A-C. Barchart representation of mean ± SEM % apoptosis induced following PK11007 treatments. Cells were stained with annexin-V (AV) and propidium iodide (PI) and analysed by flow cytometry. All experiments were carried out in triplicate. Data was analysed using Paired t-test. D-I. Barchart representation mean ± SEM fold induction of PUMA, NOXA and p21 following treatment with PK11007 compared to DMSO control. Data was analysed using Paired t-test. *p < 0.05; **p < 0.005.

**Table 1**
Top 20 Gene Ontology (GO) terms significantly enriched in differentially regulated genes in PK11007 treated HCC1143 cell lines.

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<th>GO Category</th>
<th>GO Term</th>
<th>NumDEInCat</th>
<th>NumInCat</th>
<th>P-Value</th>
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</thead>
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<tr>
<td>GO:0010941</td>
<td>Regulation of cell death</td>
<td>84</td>
<td>1330</td>
<td>2.13E-11</td>
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<tr>
<td>GO:0010033</td>
<td>Response to organic substance</td>
<td>125</td>
<td>2308</td>
<td>2.17E-11</td>
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<tr>
<td>GO:00034276</td>
<td>Regulation of programmed cell death</td>
<td>81</td>
<td>1278</td>
<td>3.97E-11</td>
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<tr>
<td>GO:00032501</td>
<td>Multicellular organisinal process</td>
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<td>1268</td>
<td>6.69E-11</td>
</tr>
<tr>
<td>GO:0003065</td>
<td>Response to external stimulus</td>
<td>261</td>
<td>6388</td>
<td>1.40E-10</td>
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<tr>
<td>GO:0003060</td>
<td>Single-multicellular organism process</td>
<td>105</td>
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<td>160</td>
<td>3543</td>
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<td>GO:0003062</td>
<td>Protein refolding</td>
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<td>21</td>
<td>1.69E-09</td>
</tr>
<tr>
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<td>Response to stimulus</td>
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<td>GO:0003064</td>
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<td>Programmed cell death</td>
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<td>GO:0003067</td>
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<td>GO:0003068</td>
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<td>Cell communication</td>
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<td>Signaling</td>
<td>223</td>
<td>5549</td>
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</table>

NumDEInCat – number of differentially expressed genes in category; NumInCat – number of genes in category.
APR-246 to reverse the mutant properties of the p53 protein [33]. With the exception of 2 genes, there was no overlap between our PK11007 regulated genes and those previously found to be modulated by APR-246 [33]. To confirm that our gene list was in fact enriched for p53 modulated genes, we compared our gene list (624 differentially regulated genes) to a curated list of mutant p53 dysregulated genes which is comprised of 668 genes [34]. We observed a higher overlap than would have been expected by chance between the genes differentially regulated by PK11007 in this investigation and those included in the curated list of mutant p53 dysregulated genes, i.e., 6.4% of our genes were p53 modulated genes (41/635) (Table 3, Suppl.) versus ~3.4% of all genes (668 genes from ~20,000 in the genome) (p-value = $1.37 \times 10^{-5}$). This finding is consistent with modulation of p53 by PK11007.

**Effect of PK11007 on refolding of mutant p53**

Unfolding of the p53 protein is one of the main consequences of structural p53 mutations. To establish if the addition of PK11007 reversed this unfolding, we used the p53 conformation-specific antibodies, PAb240 and PAb1620 for immunofluorescence staining of the treated protein. As seen in Fig. 4A, the addition of PK11007 resulted in a dose-dependent increase in staining with the wild-type associated p53 antibody, PAb1620. Simultaneously, there was a dose-dependent decrease in fluorescence using the mutant specific p53 antibody, PAb240.

To confirm these results, we quantified the fluorescent staining of PAb1620 and PAb240 by flow cytometry. A dose-dependent increase in PAb1620 fluorescence and a corresponding decrease in PAb240 fluorescence was seen in the p53 mutated cell line SKBR3. In contrast, no change PAb1620 or PAb240 fluorescence was seen in the p53-WT cell line MCF7 (Suppl. Fig. 4 A, B). Furthermore, to confirm that these changes were not due to alterations in total p53 protein, we measured the absolute p53 protein levels by ELISA. No change in absolute protein levels were seen (Suppl. Fig. 4 C). This result suggests that PK11007 altered the conformation of the mutant p53 protein by converting it to a form with wild-type-like conformation.

**Effect of PK11007 in combination with cytotoxic agents on cell line growth**

In an attempt to increase growth inhibition, PK11007 was combined with a number of clinically used chemotherapeutic drugs (Table 4, Suppl.). The effect of the drug combination on growth inhibition in the three TN cell lines investigated was both drug- and cell line-dependent. Thus, PK11007 in combination with cis-platin gave synergistic growth inhibition in two of the three cell lines
(MDA-MB-468 cells, CI = 0.6; BT549 cells, CI = 0.8; HCC1143 cells, CI = 1.3). PK11007 in combination with carboplatin, docetaxel or eribulin however, was synergistic in only one cell line. In contrast, the combination of PK11007 with doxorubicin failed to give synergistic growth inhibition in any of the 3 cell lines investigated.

**Discussion**

Our results in this study show that PK11000, PK11007 and PK11010 inhibited breast cancer cell growth in a compound- and cell line-dependent manner. Of the three compounds investigated, PK11007 was the most potent, giving the lowest IC50 values for growth inhibition. The higher potency of PK11007 than the other 2 compounds may relate to its improved membrane permeability (more hydrophobic), higher thiol reactivity (stronger electron withdrawing substituents), and increased alkylation selectivity (steric hindrance of nucleophilic substitution because of its 4-fluorobenzyl substitution).

A potentially clinically relevant finding from this work was our observation that TN cell lines were significantly more sensitive to PK11007 than the non-TN cell lines. This enhanced sensitivity in the TN cell lines may be due to the higher levels of p53 protein detected in this subset of cell lines compared with the non-TN cell lines. Indeed, across the panel of cell lines investigated, a linear inverse relationship was found between p53 protein levels and the IC50 values for PK11007, i.e., the higher the p53 protein level, the lower the IC50 Value. These finding suggest that p53 protein levels may be predictive of a preferential response to PK11007 in vivo. In contrast to the significant inverse relationship found between response to PK11007 and p53 levels, no correlation was evident between response to the compound and levels of the p53-related proteins, p63 or p73.

To further investigate the mode of action of PK11007, we investigated its impact on global gene expression. Using RNA-seq analysis, the expression of 390 genes were significantly upregulated and 234 genes downregulated following treatment with PK11007. However, we found little overlap between these altered genes and those previously reported to be altered following treatment with APR-246 [33]. These differences might be due to different modes of action of PK11007 and APR-246, different techniques used to measure gene expression (RNAseq in this investigation compared to microarray in the previously published report) or to the different target cell lines used. Indeed, as previously pointed out by Allen et al. [35], the effects of p53 on gene expression appears to be largely cell line-dependent. Despite finding little overlap between PK11007 and APR-246 regulated genes, we found substantial overlap in the genes altered by PK11007 and those previously reported to be modulated by mutant p53 [34].

Using GO analysis, several of the significantly enriched terms identified following treatment with PK11007 might be expected from the reactivation of mutant p53. These include regulation of cell death, regulation of apoptosis, protein refolding, cellular response to stimuli and signal transduction. Previously, using microarrays, Lambert et al. [33] reported that pathways involving cell-cycle arrest, apoptosis and endoplasmic reticulum stress were altered following treatment with APR-246.

Although several of the differentially regulated genes identified here had previously been linked to p53 action, several of the canonical p53 regulated genes such as NOXA, PUMA, p21, were not detected by RNA-seq in this study, following treatment with PK11007. The failure to detect altered expression in these genes using RNA-seq analysis could, in part, relate to the short exposure to the compound, i.e., 12 h, or inadequate sensitivity of the RNA-seq analysis. In this study, we deliberatively used a short treatment schedule to detect the early response genes. But, while we failed to detect altered expression of some of the canonical p53 regulated genes using RNA-seq, we found NOXA, PUMA and p21 mRNA to be upregulated by PK11007, using RT-PCR. Previously, Bauer et al. [24], found that PUMA and p21 protein levels were upregulated following treatment of gastric cancer cell lines with PK11007. Analogous to our finding with PK11007, Lambert et al. [33], using microarrays, also failed to detect upregulation of some classical p53-regulated genes such as BAX, p21 and MDM2 following treatment of Saos-His273 cells with APR-246. But, using RT-PCR, increased expression of BAX was detected. These findings suggest that global methods for detecting gene expression such microarray and RNA-seq may have inadequate sensitivity for detecting the expression of specific genes.

In conclusion, our findings that PK11007 inhibited cell proliferation and promoted apoptosis in mutant p53 cells are consistent with the ability of this compound to activate the mutant protein. But, they do not exclude the possibility that PK11007 may also be acting via other mechanisms such as increasing ROS concentration or inducing endoplasmic reticulum stress [24]. From a clinical point of view, our data suggests that PK11007 is a potential new anticancer agent, especially for TN breast cancers containing mutant p53 or high levels of p53 protein. Indeed, as p53 is the most widely mutated gene in human cancer, this compound should clearly undergo further investigations for potential anticancer activity. In breast cancer, these studies should focus on patients with TN disease.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2017.09.053.

**Conflicts of interest**

NOD and JC received research funding from Esai LTD.

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