Kevetrin™, a novel small molecule, activates p53, enhances expression of p21, induces cell cycle arrest and apoptosis in a human cancer cell line

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ABSTRACT

Kevetrin™, a small molecule currently under development, has potent antitumor activity in human multi-drug resistant carcinoma xenograft models while being well tolerated. To investigate the mechanism of action for its potent antitumor activity, we assessed Kevetrin's effect on apoptosis and cell cycle progression, including underlying molecular mechanisms.

Kevetrin strongly induced cell cycle arrest and apoptosis in a human lung adenocarcinoma cell line (A549) as shown by FACS analysis. Treatment of A549 cells with Kevetrin for 48 hours resulted in G2/M phase cell cycle arrest that was associated with a marked decline in levels of G2/M regulatory proteins, including CDK1 and cdc25B, and increased expression of Wee1, shown by western blot. It has been shown that non-genotoxic stress activates SAPK/JNK leading to degradation of cdc25B and cell cycle arrest. Our results showed that Kevetrin activated SAPK/JNK in a dose and time dependent manner. Further, Kevetrin-mediated growth inhibition of A549 correlated with apoptosis induction that was characterized by cleavage of PARP.

Reactivation of p53 in tumor cells has been recognized as a promising strategy for cancer treatment. Activation of p53 leads to cell cycle arrest and apoptosis. Kevetrin increased levels of activated p53 in A549 cells. Western blot analysis revealed a concentration dependent increase in phosphorylation of p53 at serine 15. The phosphorylation of p53 at serine 15 led to reduced interaction between p53 and its negative regulator, the oncoprotein MDM2, a ubiquitin ligase for p53 that plays the central role in stability of p53. Kevetrin also increased expression of p53 target genes such as p21(Waf1) and PUMA. The tumor suppressor protein, p21(Waf1), acts as an inhibitor of cell cycle progression whereas increased expression of PUMA induces apoptosis.

In addition to its function as transcription factor, p53 can act in the cytosol and mitochondria to promote apoptosis through a transcription-independent mechanism. Kevetrin enhanced the phosphorylation of MDM2 in a dose dependent manner and acted on the E3 processivity of MDM2. Immunoprecipitation and western blot experiments confirmed the induction of p53 monoubiquitination in response to Kevetrin. p53 monoubiquitination enhances the stability and accumulation of p53 in the cytosol or mitochondria directly activating apoptosis.

Most currently available chemotherapeutic drugs are genotoxic in nature and damage DNA. A DNA damaging drug results in rapid phosphorylation of H2A.X at Ser 139 by PI3K-like kinases. Kevetrin did not induce this phosphorylation at a concentration that caused cell cycle arrest and apoptosis; whereas, Doxorubicin did induce the phosphorylation of H2A.X, as shown by western blot assay. These results suggest that Kevetrin, in a non-genotoxic way, induces p53 activation.

Importantly, we identified the levels of p21 as a potential biomarker in our upcoming clinical trial for Kevetrin. Based on the mechanism studies, levels of p21 were measured by qPCR in peripheral blood lymphocytes from mice treated with Kevetrin. Kevetrin significantly enhanced p21 levels compared to control which correlated with anti-tumor activity of Kevetrin.

Our studies show that Kevetrin activates p53 functions in tumor cells. Thus Kevetrin is a strong candidate as an anticancer drug that targets p53. Based on our studies we plan to initiate a Phase I clinical trial in 2011.

Kevetrin has potent anti-tumor activity in human tumor xenograft models

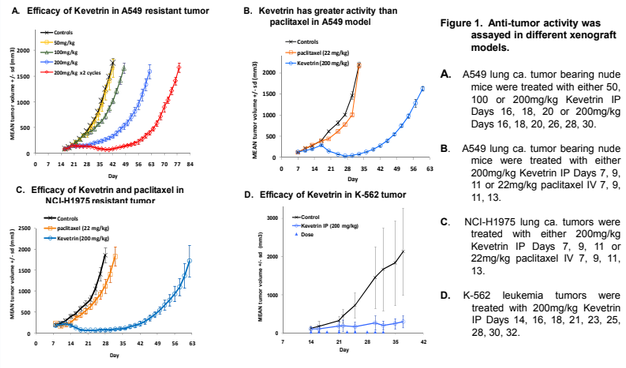


Figure 1. Anti-tumor activity was assayed in different xenograft models.

A. A549 lung ca. tumor bearing nude mice were treated with either 50, 100 or 200mg/kg Kevetrin IP Days 16, 18, 20 or 200mg/kg Days 16, 18, 20, 26, 28, 30.

B. A549 lung ca. tumor bearing nude mice were treated with either 200mg/kg Kevetrin IP Days 7, 9, 11 or 22mg/kg paclitaxel IV 7, 9, 11, 13.

C. NCI-H1975 lung ca. tumors were treated with either 200mg/kg Kevetrin IP Days 7, 9, 11 or 22mg/kg paclitaxel IV 7, 9, 11, 13.

D. K-562 leukemia tumors were treated with 200mg/kg Kevetrin IP Days 14, 16, 18, 21, 23, 25, 28, 30, 32.

Kevetrin enhanced G2/M cell cycle arrest associated with marked changes in levels of G2/M regulatory proteins

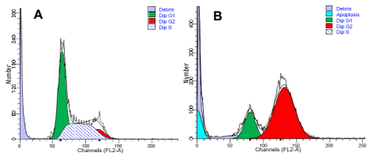
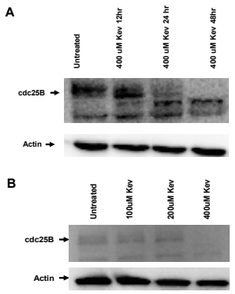


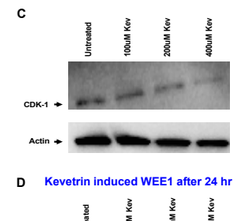
Figure 2. Cell cycle profile of A549 cells. Cell-cycle changes examined by propidium iodide staining.
A. untreated **B**. treated with 400μM Kevetrin for 42 hr.

Regulatory proteins for CDK1 function

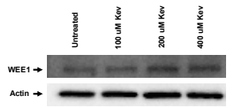
G2/M arrest was associated with a degradation of cdc25B protein after treatment with Kevetrin



G2/M arrest was associated with decreased CDK1 protein after treatment with Kevetrin



Kevetrin induced WEE1 after 24 hr



Kevetrin activated SAPK/JNK (stress-activated protein kinase/jun-amino-terminal kinase)

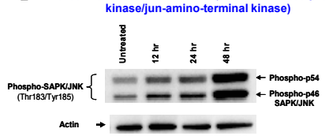


Figure 3. Western blot analysis of extracts of A549 cells untreated or treated with Kevetrin for changes in the level of expression of G2/M regulatory proteins detected by immunoblot. Extract from A549 cells treated or untreated with Kevetrin for indicated times and concentrations were immunoblotted with different antibodies.

A & B. cdc25B rabbit mAb **C.** CDK1 mouse mAb **D.** WEE 1 rabbit mAb **E.** SAPK/JNK rabbit mAb

Kevetrin induced PARP and Caspase3 cleavage

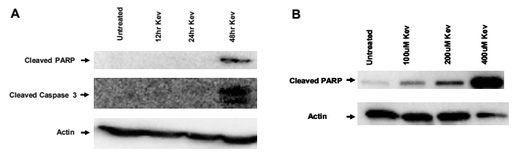


Figure 4. Western blot analysis of extracts of A549 cells untreated or treated with Kevetrin.

A. A549 cells were treated with 400μM of Kevetrin and immunoblotted with cleaved PARP(Asp214) rabbit mAb and cleaved caspase3 (Asp175) rabbit mAb.

B. A549 cells were treated for 48 hr with Kevetrin and immunoblotted with cleaved PARP(Asp214) rabbit mAb .

Kevetrin induced p53 expression

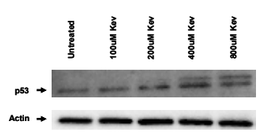


Figure 5. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using p53 antibody.

Kevetrin induced phosphorylation of p53 (Serine 15)

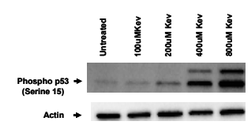


Figure 6. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using phospho-p53(ser15) antibody.

Kevetrin induced p21 Waf1/Cip expression

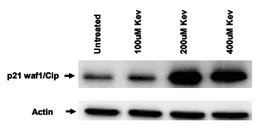


Figure 7. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using p21Waf1/Cip rabbit mAb.

Kevetrin induced PUMA expression

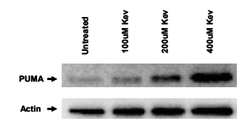
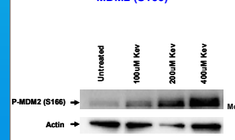


Figure 8. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using PUMA rabbit mAb.

Kevetrin induced phosphorylation of MDM2 (S166)



Kevetrin acts on E3 processivity of MDM2 and monoubiquitinates p53

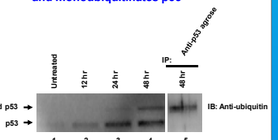


Figure 9. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using phospho-MDM2(S166) rabbit mAb.

Figure 10. Western blot analysis of extracts from A549 cells untreated or treated with 400 μM Kevetrin for indicated time using p53 antibody (lanes 1 - 4). For lane 5, extract from Kevetrin treated for 48 hr was immunoprecipitated (IP) with p53 antibody. Ubiquitinated p53 was detected by immunoblotting (IB) with anti-ubiquitin antibody.

Kevetrin did NOT induce the phosphorylation of H2A.X indicating that Kevetrin is non-genotoxic

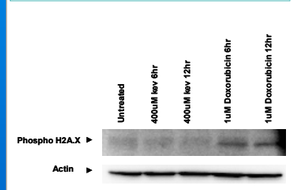


Figure 11. Western blot analysis of extracts from A549 cells untreated or treated with 400 μM of Kevetrin and 1 μM of Doxorubicin at indicated times using phopho H2A.X rabbit mAb.

Expression levels of p21 in lymphocytes is a potential bio-marker for Kevetrin

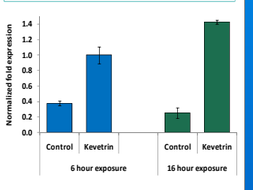
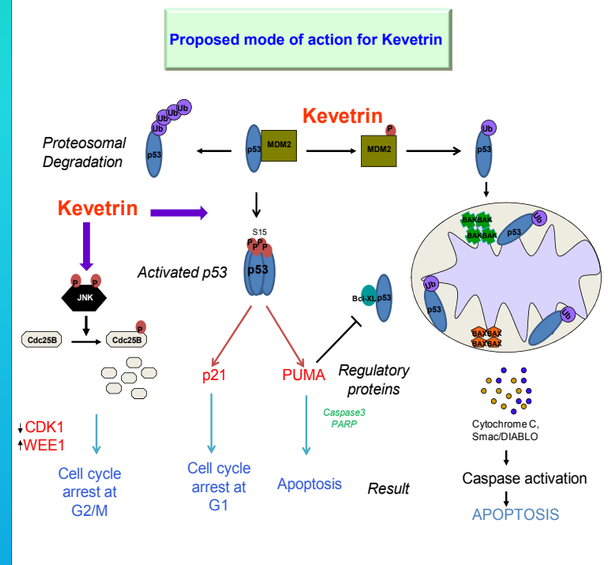


Figure 12. Real time PCR analysis of p21 gene expression. Mice were injected with 200mg/kg IP of Kevetrin. Blood samples were collected at 6 or 16 hrs after injection. Lymphocytes were isolated and RNA was extracted. Expression levels of p21 were measured using qPCR.

Proposed mode of action for Kevetrin



CONCLUSION

Our results show that Kevetrin activated both transcription-dependent and transcription-independent pathways to promote cell cycle arrest and apoptosis through p53 activation in tumor cells. Apoptosis induced by p53 is established as a central mechanism of tumor suppression; activation of both pathways of p53 by Kevetrin has far-reaching significance in cancer therapy.

By activating wild type p53, tumor regression is expected since p53 acts as a functional transcriptional factor for tumor suppression. In tumors that retain wild type p53, its activity can be partially abrogated through the inactivation of signaling or effector molecules. Inactivation of p53 by mutation occurs in 50% of human cancers; however, the transcription mediated response of activated mutant p53 does not necessarily lead to apoptosis. Since Kevetrin acts via a transcription-independent mechanism by altering the E3 processivity of MDM2, the mutant p53 can induce apoptosis. Recently, it has been shown that tumors with p53 mutations retain pro-apoptotic activity through transcription independent mechanisms.

Kevetrin showed potent efficacy in many mutant p53 tumor xenografts e.g. MDA-MB-231 (breast ca.), HT-29 (colon ca.), NCI-H1975 (lung ca.), HCT-15 (colon ca.) (data not shown). We found that stable monoubiquitinated mutant p53 was induced by Kevetrin. This form of p53 has been shown to accumulate in the cytoplasm and mitochondria and retain the ability to interact with BAK or BAX proteins in mitochondria to induce apoptosis. Thus Kevetrin can function as a major inducer of apoptosis in many types of tumors. Activation of both modes of apoptosis by Kevetrin may not be mutually exclusive. Most likely, both modes of apoptosis induction cooperate and complement each other.

Since Kevetrin targets both wild type and mutant p53, has potent efficacy in xenograft models, and was well-tolerated in GLP safety pharmacology and toxicity studies, we plan to initiate a Phase I clinical trial in 2011.

For further information

Please contact *info@cellceutix.com* 978-921-4180. More information on this and related projects can be obtained at *http://www.cellceutix.com/*

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