Kevetrin™ targets both MDM2-p53 and Rb-E2F pathways in tumor suppression

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ABSTRACT

Our studies showed that Kevetrin™, a small molecule currently under development, has potent antitumor activity in several wild type and mutant p53 human tumor xenografts, e.g., A549, MIA PaCa-2, PC-3, MDA-MB-231, HT-29, NCI-H1975, HCT-15, K-562, LNCaP. To investigate the mechanism of action of its antitumor activity in different xenograft models, we assessed Kevetrin's effects on apoptosis and cell cycle progression.

HDAC inhibitors/regulators mediate cell death through several pathways. HDAC1 and HDAC2 are deregulated in many cancers and are the main deacetylases involved in numerous cancer types. Downregulation of HDAC2 has been shown to inhibit tumor growth. Kevetrin downregulated HDAC2 and HDAC6 in many mutant p53 and null p53 cancer cell lines. Kevetrin acts through alterations in chromatin modification and gene expression, modification of the expression and function of the HDAC6-chaperone axis and modulation of transcription factor expression.

Our microarray expression study showed several-fold decreases in HDAC2 and HDAC6 expression in response to Kevetrin. Kevetrin strongly induced apoptosis in multiple tumor cell lines characterized by activation of PARP. Kevetrin upregulated pro-apoptotic proteins, including PUMA which was observed in A549 and BID which was observed in MIA PaCa-2 and MDA-MB-231 cell lines. Kevetrin induced downregulation of anti-apoptotic protein Mcl-1 in MDA-MB-231 cell line. Kevetrin induced cell cycle arrest via induction of cyclin-dependent kinase inhibitor p21 in many p53 wild type, mutant and null cell lines. In wild type p53 human lung carcinoma (A549), Kevetrin showed a concentration dependent increase in activation of p53 by Western blot analysis. Kevetrin induced apoptosis in a transcriptional independent way by altering the E3 ligase processivity of MDM2. Immunoprecipitation and Western blot experiments confirmed the induction of p53 monoubiquitination in response to Kevetrin. p53 monoubiquitination enhanced the stability and accumulation of p53 in the cytosol or mitochondria directly activating apoptosis.

In many mutant p53 tumors, gain of function and hyperstability of p53 drives tumor formation, invasion and metastasis. Kevetrin downregulated mutant p53 in MDA-MD-231 and MIA PaCa-2 cell lines. The downregulation of mutant p53 may be associated with Kevetrin induced inhibition of HDAC6-Hsp90 chaperone axis. Kevetrin by virtue of depleting mutant p53 may be able to dramatically chemosensitize mutant p53 cells to chemotherapeutic drugs.

E2F1 overexpression, observed in most tumors, is associated with tumor growth. Kevetrin down regulated E2F1 expression in various p53 wild type and mutant cell lines. Kevetrin also down regulated the E2F1 target gene thymidylate synthase (TS) in various tumor cell lines. Effective inhibition of TS by Kevetrin could play a crucial role in tackling the problem of resistance.

We have also demonstrated that Kevetrin is non-genotoxic. DNA damaging drugs result in rapid phosphorylation of H2A.X at Ser 139 by PI3K-like kinases; however, Kevetrin did not induce phosphorylation of H2A.X.

Kevetrin was well-tolerated in GLP safety pharmacology and toxicity studies, and has shown unique mechanism of action with potent anti-tumor activity while being non-genotoxic; therefore, we have submitted an IND application for a Phase I clinical trial.

Kevetrin has potent anti-tumor activity in xenograft models with varied p53 mutant status

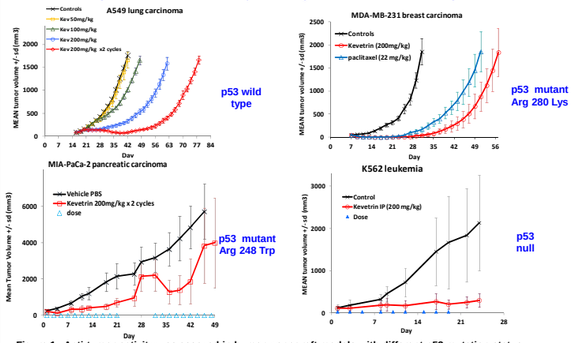


Figure 1. Anti-tumor activity was assayed in human xenograft models with different p53 mutation status.

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

MIA-PaCa-2 pancreatic ca. tumor bearing nude mice were treated with 200mg/kg Kevetrin IP Days 1 to 21 and 32 to 52 every other day after tumors were established.

MDA-MB-231 breast 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 after tumor implantation.

K-562 leukemia tumors were treated with 200mg/kg Kevetrin IP Days 1,3,5, 8,10,12, 15,17,19 after tumors were established.

GLP Toxicology and Safety Pharmacology Studies of Kevetrin

Repeat dose toxicology studies: IV weekly x 5 weeks

- Rats (60, 90, 120 mg/kg) NOAEL = 90 mg/kg
  - o minor changes in hematology, clinical chemistries
- Dogs (5, 25, 60 mg/kg) NOAEL = 5 mg/kg
  - o vomiting, loose stool, diarrhea (during dosing only)
  - o biologically significant decrease in MCHC and increases in RBC, hematocrit, and sodium (high dose)

Cardiovascular effects: 60 mg/kg (dogs)

- No effect on ECG
- Transient increase in heart rate, arterial pressure, diastolic pressure – resolved within 7 hours after dosing

Neurological effects: 120 mg/kg IV (rats)

- No apparent neuropharmacological, temperature effects

Pulmonary effects: 120 mg/kg IV (rats)

- No biologically relevant effects on respiratory rate, tidal volume or minute volume

Kevetrin's effects on PARP, HDAC2, E2F1, thymidylate synthase

p53 status	Cell line	Organ	Mutation	PARP cleavage	HDAC2	E2F1	Thymidylate synthase
wild type	A549	lung	WT	+	⇩	⇩	?
	U-87 MG	brain	WT	+	⇩	⇩	*n.d.*
	LNCaP	prostate	WT	+	⇩	*n.d.*	*n.d.*
	MM.1S	multiple myeloma	WT	+	⇩	*n.d.*	*n.d.*
mutant	MIA PaCa-2	pancreas	Arg 248 Trp	+	⇩	⇩	*n.d.*
	NCI-H1975	lung	Arg 273 His	+	⇩	⇩	*n.d.*
	HT-29	colon	Arg 273 His	+	⇩	*n.d.*	*n.d.*
	MDA-MB-231	breast	Arg 280 Lys	+	⇩	*n.d.*	*n.d.*
	U266B1	B lymphocyte	Ala 161 Thr	+	⇩	⇩	⇩
	RPMI 8226	B lymphocyte	Gly 285 Lys	+	⇩	⇩	⇩
null	K-562	bone marrow	Null	+	⇩	⇩	⇩
	PC-3	prostate	STOP 163	+	⇩	*n.d.*	*n.d.*

⇩ down-regulated
\+ positive
n.d. not done

Kevetrin down regulated HDAC2 expression in MDA-MB-231 and K-562 cells

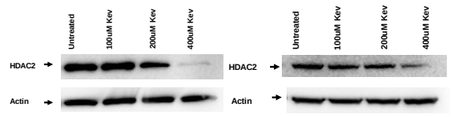


Figure 2. Western blot analysis of extracts from MDA-MB-231 and K-562 cells untreated or treated with Kevetrin for 48hr using HDAC2 mouse mAb.

Kevetrin down regulated expression of HDAC6 in MIA PaCa-2 cells

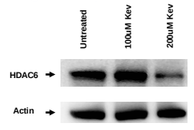


Figure 3. Western blot analysis of extracts from MIA PaCa-2 cells untreated or treated with Kevetrin for 48hr using HDAC6 rabbit mAb.

No significant change in expression of HDAC1 was observed in MIA PaCa-2 or A549 cells

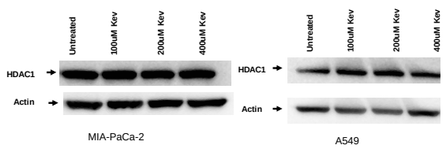


Figure 4. Western blot analysis of extracts from MIA PaCa-2 and A549 cells untreated or treated with Kevetrin for 48hr using HDAC1 mouse mAb.

Kevetrin induced p21Waf1/Cip expression in MIA PaCa-2 and MDA-MB-231 cells

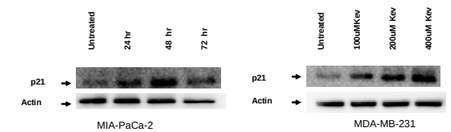


Figure 5. Western blot analysis of extracts from MIA PaCa-2 and MDA-MB-231 cells untreated or treated with Kevetrin using p21Waf1/Cip rabbit mAb. MIA PaCa-2 cells were treated with 400μM of Kevetrin over 3 time points. MDA-MB-231 cells were treated for 48 hr at 3 concentrations.

Kevetrin down regulated E2F1 expression in MDA-MB-231 and MIA PaCa-2 cells

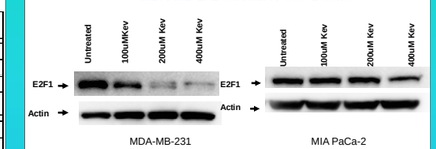


Figure 6. Western blot analysis of extracts from MDA-MB-231 and MIA PaCa-2 cells untreated or treated with Kevetrin for 48hr using E2F1 mouse mAb.

Kevetrin down regulated thymidylate synthase expression in K-562 cells

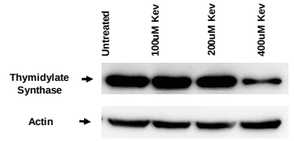


Figure 7. Western blot analysis of extracts from K-562 cells untreated or treated with Kevetrin for 48hr using thymidylate synthase rabbit mAb.

Kevetrin induced cleavage of BH3-only protein BID in MIA PaCa-2 and MDA-MB-231 cells

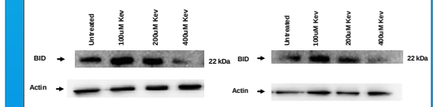


Figure 8. Western blot analysis of extracts from MIA PaCa-2 and MDA-MB-231cells untreated or treated with Kevetrin for 48hr using BID rabbit polyclonal Ab.

Kevetrin down regulated the Mcl-1, an anti-apoptotic member of the Bcl-2 family protein

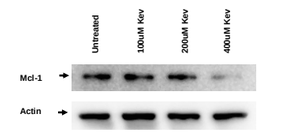


Figure 9. Western blot analysis of extracts from MDA-MB-231 cells untreated or treated with Kevetrin for 48hr using Mcl-1 rabbit mAb.

Kevetrin enhanced acetylation of histone H3 protein in A549 cells

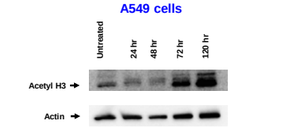


Figure 10. Western blot analysis of extracts from A549 cells untreated or treated with 400 μM Kevetrin over 4 time points using Acetyl Histone H3 rabbit polyclonal Ab.

Kevetrin enhanced the acetylation of α-tubulin (Lys40) in MDA-MB-231 cell lines

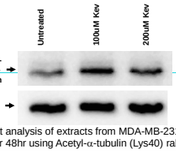


Figure 11. Western blot analysis of extracts from MDA-MB-231 cells untreated or treated with Kevetrin for 48hr using Acetyl-α-tubulin (Lys40) rabbit mAb.

Kevetrin down regulated and/or degraded mutant oncogenic p53 in MDA-MB 231 cells

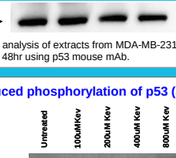


Figure 12. Western blot analysis of extracts from MDA-MB-231 cells untreated or treated with Kevetrin for 48hr using p53 mouse mAb.

Kevetrin induced phosphorylation of p53 (Serine 15)

Figure 13. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using phospho-p53 (ser15) rabbit mAb.

Kevetrin induced phosphorylation of MDM2 (S166)

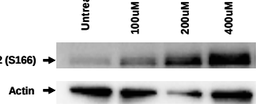


Figure 14. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using phospho-MDM2 (S166) rabbit mAb.

Kevetrin acts on E3 processivity of MDM2 and monoubiquitinates p53

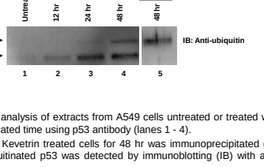


Figure 15. Western blot analysis of extracts from A549 cells untreated or treated with 400 μM Kevetrin for indicated time using p53 antibody (lanes 1 - 4).

For lane 5, extract from Kevetrin treated cells 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

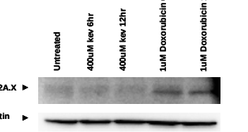
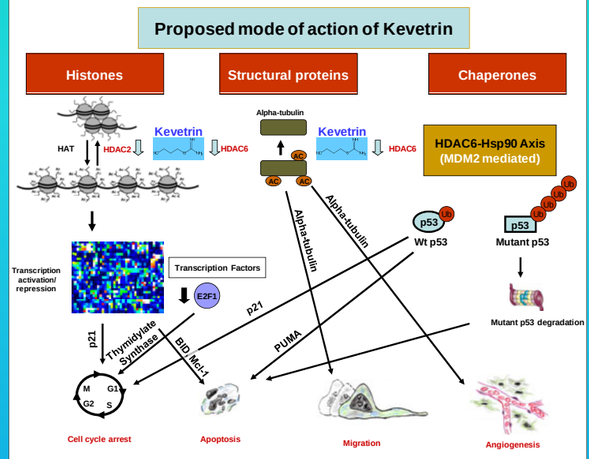


Figure 16. Western blot analysis of extracts from A549 cells untreated or treated with 400 μM of Kevetrin or 1 μM of Doxorubicin at indicated times using phopho H2A.X rabbit mAb.

Proposed mode of action of Kevetrin



CONCLUSION

Due to the complexity of pro-apoptotic and anti-apoptotic pathways with multiple players involved and redundant signaling networks, blocking only one anti-apoptotic factor may not result in antitumor activity. However, Kevetrin acts via multiple targets to produce efficacy in a wide range of xenograft tumor models.

Kevetrin has potent antitumor activity in several wild type and mutant p53 human tumor xenografts, e.g., A549, MIA Paca-2, MDA-MB-231, K-562, PC3, HT-29, NCI-H1975, HCT-15 and LNCaP. Kevetrin modulated the expression of HDAC2 and HDAC6. HDAC inhibition/regulation has been shown to mediate cell death through several pathways, including cell cycle arrest, induction of apoptosis, anti-angiogenesis and affects misfolded protein response pathways. Kevetrin acts through alterations in chromatin modification and gene expression, modification of the expression and function of the HDAC6-chaperone axis and modulation of transcription factor expression. Kevetrin induced apoptosis by upregulation of pro-apoptotic protein expression and downregulation of anti-apoptotic proteins in different cell lines. Kevetrin induced cell cycle arrest via induction of p21 in many p53 wild type, mutant and null cell lines.

In recent years, HDAC has emerged as a promising target for therapeutic interventions that could revert the aberrant epigenetic states associated with cancer. Overexpression of HDAC1 and HDAC2 have been reported in many types of cancer. Downregulation of HDAC2 by Kevetrin induced upregulation of specific pro-apoptotic genes and/or downregulation of prosurvival genes, therefore reactivating pathways controlling apoptosis or cell growth.

Rb-E2F and MDM2 p53 pathways are crucial regulators of cell cycle progression. E2F1 overexpression, observed in most tumors, is associated with tumor growth. Kevetrin downregulated E2F1 and its target gene, thymidylate synthase, in A549, MIA PaCa-2, K-562, and NCI-H1975 cell lines. In xenograft models with these tumors, Kevetrin had very potent anti-tumor activity. Transcription factor p53 is a major tumor suppressor and its stability determines whether it has the capacity to carry out its task. MDM2 is the E3 ubiquitin ligase that promotes ubiquitin-dependent proteosomal degradation of p53. Kevetrin activates and stabilizes the wild type p53 by monoubiquitination. Stable monoubiquitinated p53 accumulation in cytosol or mitochondria directly activates apoptosis. Kevetrin degrades mutant p53 in MDA-MB-231 and other mutant cell lines. Degradation of mutant p53 by Kevetrin can chemosensitize mutant p53 cancer cells to chemotherapeutic drugs. Kevetrin acting through both major pathways of tumor suppression, Rb-E2F and MDM2-p53, has far reaching consequences.

Kevetrin's multi-targeted mechanism likely contributes to its potent anti-tumor activity in various tumor types with varied p53 mutation status. This unique selectivity in turn supports Kevetrin's low toxicity profile observed during GLP safety pharmacology and toxicity studies. We plan to initiate a Phase I clinical trial at Dana-Farber/Harvard Cancer Centers in 2012.

For further information

Please contact *info@cellceutix.com* 978-921-4180.

More information on this and related projects can be obtained at *www.cellceutix.com*.

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