Discovery and characterization of Kevetrin™: A small molecule with potent anti-cancer activity

Tejindervir Singh Hiran, Karima Chafai-Fadela, Samantha Rogers, Ashok Kumar, Sylvia Holden, Siya Ram, Krishna Menon.

***Cellceutix Pharmaceuticals** (a publicly traded company, stock ticker: CTIX) Beverly, MA, USA*

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ABSTRACT

Kevetrin™, a new chemical entity, possesses a broad spectrum of anticancer activity, and is currently under development. Kevetrin™ has been found efficacious in human multi-drug resistant lung carcinoma xenograft models with minimal toxicity and is not genotoxic.

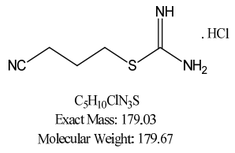
Mechanistically, Kevetrin strongly induced cell cycle arrest and apoptosis in a human lung adenocarcinoma cell line (A549). Furthermore, Kevetrin-mediated growth inhibition of A549 correlated with apoptosis induction that was characterized by cleavage of procaspase-3 and poly (ADP-ribose) polymerase (PARP). Interestingly, Kevetrin treatment caused enhanced activated p53 levels in A549 cells. Activation of p53 can lead to cell cycle arrest and apoptosis. Western blotting revealed a concentration dependent increase in phosphorylation of p53 at serine 15. The phosphorylation of p53 at serine 15 leads to reduced interaction between p53 and its negative regulator, the oncoprotein MDM2. Kevetrin also increases expression of p53 target genes such as p21 (Waf1), which acts as inhibitor of cell cycle progression.

Kevetrin acts via a transcription-independent mechanism by altering the E3 processivity of MDM2, thereby stabilizing p53 which can induce apoptosis. Recently, it has been shown that tumors with p53 mutations retain pro-apoptotic activity through transcription independent mechanisms.

The pharmacokinetic profile of Kevetrin showed a biphasic curve for plasma concentration vs. time, and had an elimination half-life of 0.7 hours in rats and 1.0 hours in dogs. Toxicological studies suggest that Kevetrin had minor adverse effects and was well tolerated. These findings led to the advancement of Kevetrin™ into a Phase I clinical trial planned for 2012.

INTRODUCTION

Lung cancer is a leading cause of death in men and women and is responsible for 160,000 deaths annually in the United States. Non-small-cell lung cancer accounts for 80% of bronchogenic neoplasms with 95% of patients dying within five years of diagnosis; therefore, development of novel small molecules for the treatment of lung cancer is a highly desirable goal.



$C_5H_{10}ClN_3S$
Exact Mass: 179.03
Molecular Weight: 179.67

Figure 1. Chemical Structure of Kevetrin

In general, commonly used chemotherapeutic agents act by killing continuously dividing tumor cells. These agents also harm normal cells that divide rapidly such as bone marrow cells, digestive tract and hair follicles that result in side effects such as myelosuppression, mucositis and alopecia. In our on-going efforts to develop a novel anti-cancer agent for the treatment of lung cancer that targets tumor cells rather than being cytotoxic, like currently used chemotherapeutic agents, we have developed Kevetrin™, S-(3-cyanopropyl)isothiourea hydrochloride salt as shown in Figure 1. This novel compound has demonstrated a broad spectrum of anticancer activity in human xenograft tumor models with minimal toxicity.

Kevetrin Analogues

Kevetrin can be easily prepared by a reaction of 4-chlorobutyronitrile and thiourea with good yield. Several analogs were prepared in a similar fashion as described above to explore preliminary structure activity relationships. These compounds were characterized by spectral and analytical data, and initially evaluated for their cellular activity.

human solid tumor cell lines
- lung carcinoma (A549)
- breast carcinoma (MD-MBA-231)
- colon adenocarcinoma (HT-29)
- pancreatic carcinoma (MIA PaCa-2)

human hematopoietic cancer cell lines
- chronic myelogenous leukemia (K-562)
- myeloma cell lines (MM.1S, RPMI 8226, U266B1)

	IC_{50} (μM)[a]			
Cell lines	$NC(CH_2)_2$ $SC(=NH)NH_2$	Kevetrin $NC(CH_2)_3$ $SC(=NH)NH_2$	$NC(CH_2)_4$ $SC(=NH)NH_2$
A549	>1,000	788	>1,000
MDA-MB-231	>1,000	421	695
HT-29	>1,000	>1,000	>1,000
MIA PaCa-2	>1,000	730	>1,000
K-562	>1,000	>1,000	>1,000
U266B1	294	186	540
RPMI 8226	266	119	325
MM.1S	164	47	290

Table 1. Effects of Kevetrin and analogs on the viability of human solid tumor and hematopoietic cancer cell lines.
[a]Cellular viability was measured using a tetrazolium based assay after 48 hr exposure to Kevetrin.

	IC_{50} (μM)[a]				
Tumor Cell Lines	**Kevetrin**	**Cisplatin**	**Paclitaxel**	**5-FU**
A549	969 ± 151	84 ± 52	91 ± 68	1302 ± 505
NCI-H1975	250 ± 37	15 ± 3.4	5.7 ± 4.9	69 ± 28
MDA-MB-231	210 ± 49	10 ± 4.2	0.01 ± 0.1	14 ± 7.1
MDA-MBA-435S	1133 ± 354	11 ± 0.8	2.7 ± 0.01	4.2 ± 0.9
HT-29	1397 ± 390	63 ± 22	4.8 ± 2.3	30 ± 7.3
SCC-15	370 ± 24	4.4 ± 1.1	0.56 ± 0.48	767 ± 3035

Table 2. Effects of Kevetrin and chemotherapeutic agents on the viability of human tumor cell lines.
[a]Cellular viability was measured using a tetrazolium based assay after 48 hr exposure to Kevetrin.

Cellular viability data of Kevetrin and two of the isothiourea derivatives are illustrated in *Table 1*.

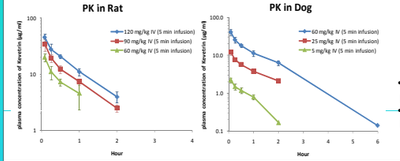
•Preliminary structure activity relationships data suggest that a 3 carbon chain between the nitrile group and sulfur is generally more potent than 2 or 4 carbon chains.

•Kevetrin (3 carbon chain) demonstrated greater activity in myeloma cell lines (U266B1, RPMI 8226 and MM.1S) than in solid tumor cell lines (A549, MDA-MB-231 and MIA PaCa-2).

The effect of Kevetrin on cellular viability was also compared with three commonly used chemotherapeutic agents: cisplatin (alkylating agent), paclitaxel (taxane) and 5-fluorouracil (anti-metabolite) in six human solid tumor cell lines after 48 hr exposure.

The results from this study demonstrated that Kevetrin was markedly *less* potent in reducing cell viability than was cisplatin, paclitaxel and 5-fluorouracil as illustrated in *Table2*.

Pharmacokinetics



Kevetrin IV	
	Elim $t_{1/2}$
rat	0.7 ± 0.4 hr
dog	1.0 ± 0.2 hr

- dose dependent increases in plasma concentration, AUC_{all}, C_{max}
- undetectable concentrations by 24 hours, low risk of accumulation

Kevetrin was effective in multi-drug resistant lung tumor xenograft models

When Kevetrin and paclitaxel, which is commonly used to treat lung cancer, were evaluated for *in vivo* efficacy in two human lung xenograft tumor models (NCI-H1975 & A549), Kevetrin had more potent antitumor activity than paclitaxel at approximately equitoxic doses, based on weight loss (*Figure 3*). A549, which have wild-type p53 but have the ras mutation (K-12), and NCI-H1975, which have mutant p53, are both multi-drug resistant tumor cells. These findings suggest that Kevetrin *in vivo* may be acting through specific signal transduction pathway(s).

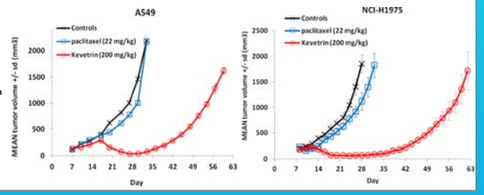


Figure 3: Efficacy of Kevetrin and Paclitaxel in A549 (left) and NCI-H1975 (right) xenograft models.

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

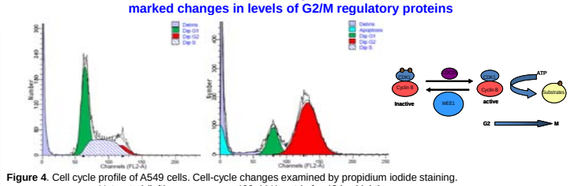


Figure 4. Cell cycle profile of A549 cells. Cell-cycle changes examined by propidium iodide staining.
Untreated (left) 400μM Kevetrin for 42 hr. (right)

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

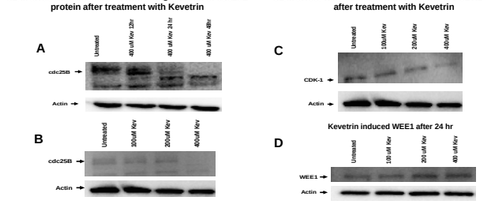


Figure 5. Western blot analysis of extracts of A549 cells untreated or treated with Kevetrin for changes in the level of expression of G2/M regulatory proteins detected by immunoblot. Extract from A549 cells treated or untreated with Kevetrin for indicated times and concentrations were immunoblotted with different antibodies.

A & B. cdc25B rabbit mAb **C.** CDK1 mouse mAb **D.** WEE 1 rabbit mAb

Kevetrin induced PARP and Caspase3 cleavage

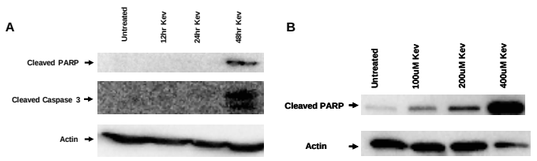
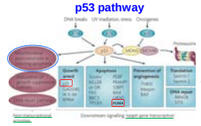


Figure 6. Western blot analysis of extracts of A549 cells untreated or treated with Kevetrin.

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.

p53 pathway



Brown E.J et. al. 2009 Nature Review Cancer 9:862-73

Kevetrin induced phosphorylation of p53 (Serine 15) | **Kevetrin induced p21 Waf1/Cip expression** | **Kevetrin induced PUMA expression**

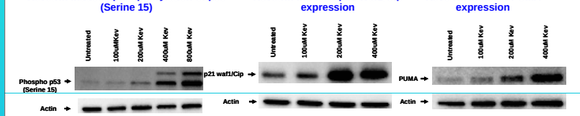


Figure 6. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using phospho-p53(ser15) antibody (left), p21Waf1/Cip rabbit mAb (center), or PUMA rabbit mAb (right).

Kevetrin induced phosphorylation of MDM2 (S166) | **Kevetrin acts on E3 processivity of MDM2 and monoubiquitinates p53**

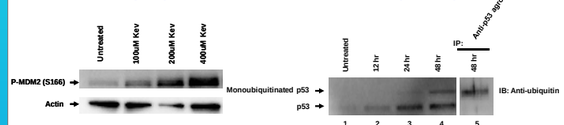


Figure 7. Western blot analysis of extracts from A549 cells untreated or treated with Kevetrin for 48hr using phospho-MDM2(S166) rabbit mAb (left) or with 400 μM Kevetrin for indicated time using p53 antibody (lanes 1 - 4). For lane 5, extract from Kevetrin treated for 48hr was immunoprecipitated (IP) with p53 antibody. Ubiquitinated p53 was detected by immunoblotting (IB) with anti-ubiquitin antibody.

Mechanism of action

Mechanism of action studies showed that Kevetrin strongly induced apoptosis, characterized by activation of Caspase 3 and cleavage of PARP. p53 is an important tumor suppressor that acts to restrict proliferation by inducing cell cycle checkpoints, apoptosis or cellular senescence. Activation of p53 also induces apoptosis by inducing the expression of p53 target gene PUMA.

- Kevetrin (400 μM) increased levels of activated p53 in A549 cells
- A concentration dependent increase in phosphorylation of p53 at serine15 was observed.
  - o This reduced interaction between p53 and its negative regulator, MDM2, a ubiquitin ligase for p53 that plays the central role in stability of p53.
- Kevetrin increased expression of p53 target genes: p21(Waf1) (cell cycle inhibitor), PUMA (induces apoptosis)

In addition to its function as a transcription factor, p53 can act in the cytosol and mitochondria to promote apoptosis through a transcription-independent mechanism.

- Kevetrin enhanced phosphorylation of MDM2 in a dose dependent manner and acted on the E3 processivity of MDM2.
- Kevetrin induced p53 monoubiquitination
  - o Monoubiquitination of p53 enhances stability and accumulation of p53 in the cytosol or mitochondria, directly activating apoptosis.

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

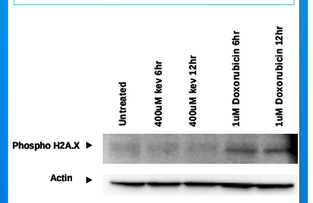


Figure 8. Western blot analysis of extracts from A549 cells untreated or treated with 400 μM of Kevetrin and 1 μM of Doxorubicin at indicated times using phospho H2A.X rabbit mAb.

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

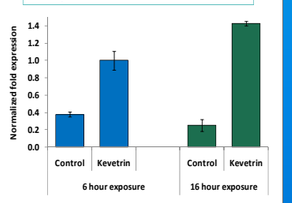


Figure 9. Real time PCR analysis of p21 gene expression. Mice were injected with 200mg/kg IP of Kevetrin. Blood samples were collected at 6 or 16 hrs after injection. Lymphocytes were isolated and RNA was extracted. Expression levels of p21 were measured using qPCR.

Toxicology and Safety Pharmacology of Kevetrin

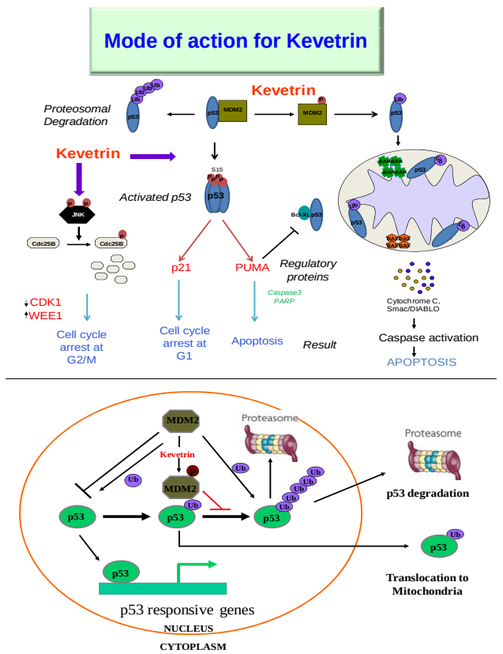
Repeat dose toxicology studies: IV weekly x 5 weeks
- Rats (60, 90, 120 mg/kg) NOAEL = 90 mg/kg
  - o minor changes in hematology, clinical chemistries
- Dogs (5, 25, 60 mg/kg) NOAEL = 5 mg/kg
  - o vomiting, loose stool, diarrhea (during dosing only)
  - o biologically significant decrease in MCHC and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

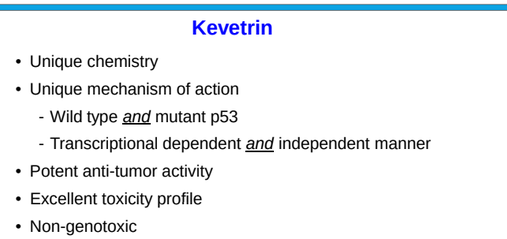
Cardiovascular effects: 60 mg/kg (dogs)
- No effect on ECG
- Transient increase in heart rate, arterial pressure, diastolic pressure – resolved within 7 hours after dosing

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

Mode of action for Kevetrin





Kevetrin

- Unique chemistry
- Unique mechanism of action
  - Wild type *and* mutant p53
  - Transcriptional dependent *and* independent manner
- Potent anti-tumor activity
- Excellent toxicity profile
- Non-genotoxic

CONCLUSION

We have developed a novel anticancer agent that is well-tolerated. Kevetrin (200 mg/kg) showed potent efficacy in many mutant p53 tumor xenografts e.g. A549, NCI-H1975 (lung ca.), MDA-MB-231 (breast ca.), HT-29 (colon ca.), HCT-15 (colon ca.) (data not shown).

Stable monoubiquitinated 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.

The toxicity profile of Kevetrin demonstrated that it was well-tolerated in doses at or above equivalent doses used in efficacy studies. In addition, lack of phosphorylation of H2A.X at Ser139 by PI3K-like kinases suggest that Kevetrin is non genotoxic.

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 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. Cellceutix is a publicly traded company, stock ticker: CTIX

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