AVA6000, a novel preCISION medicine, targeted to the tumor microenvironment via Fibroblast Activation Protein (FAP) mediated cleavage

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Introduction

AVA6000 is a therapeutic product based on proprietary pre|CISION™ technology which incorporates a substrate that is sensitive to cleavage by FAP. The pre|CISION™ substrate can be utilised in a drug conjugate linker or to generate chemotherapy prodrugs that are only activated in the tumor microenvironment. AVA6000 consists of a doxorubicin molecule covalently bonded to a dipeptide (pyridine-4-carbonyl)-D-Ala-L-Pro), which is designed to be susceptible to hydrolysis by Fibroblast Activation Protein α (FAP) but is resistant to hydrolysis by both closely related and wider mammalian peptidases.

The preCISION™ Platform and AVA6000

The primary mechanism of action of doxorubicin is thought to involve stabilisation of a topoisomerase-II-DNA cleavable complex through non-specific DNA-intercalation. The non-specific DNA-intercalation causes a number of downstream effects, which may ultimately result in apoptotic cell death. Although doxorubicin has been one of the most effective and widely used chemotherapeutic agents for the treatment of various solid malignancies for over 40 years, its clinical utility is limited by dose-limiting toxicities, including myelosuppression and cardiotoxicity.

The unique FAP specificity of the N-(pyridine-4-carbonyl)-D-Ala-L-Pro leaving group conjugated to doxorubicin in AVA6000 is supported by the absence of cleavage of the fluorogenic analogue, 3114-AMC, in FAP gene-knockout mice ($Fap^{-/-}$). In vitro cytotoxicity assessments involving human tumor cell lines showed that AVA6000 was between 80-fold to 4,000-fold less cytotoxic compared to doxorubicin. In several in vivo efficacy studies in tumours with high FAP levels, AVA6000 significantly decreased tumor volume and increased survival in a dose-dependent manner. In a PDX model of osteosarcoma, AVA6000 significantly decreased tumor volume while doxorubicin had no significant effect.

FAP mediated hydrolysis of AVA6000 releases free Doxorubicin

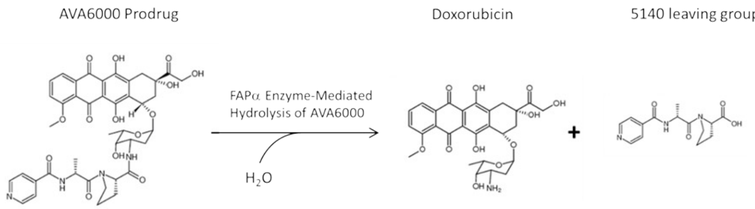


Figure 1: *The peptide bond between the carboxy-terminal proline of the oligopeptide, N-(pyridine-4-carbonyl)-D-Ala-L-Pro and the amino group of daunosamine in doxorubicin is intended to be selectively cleaved by FAP.*

AVA6000 is selectively cleaved by FAP vs other family members

Enzyme	Doxorubicin concentration (μM ± SEM)
DDP2	Not detected
DPP4	Not detected
DPP8	Not detected
DPP9	Not detected
FAP	0.23 ± 0.01
PREP	Not detected

Table 1: *Mean doxorubicin concentrations (n = 3) were measured by LC-MS/MS after incubation with the indicated recombinant enzymes. Each enzyme (5 nM final concentration) was incubated with AVA6000 (1 μM final concentration) for 30 min at 37°C.*

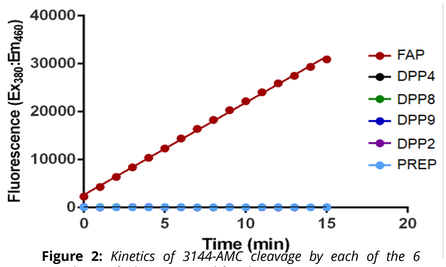


Figure 2: *Kinetics of 3144-AMC cleavage by each of the 6 members of the DASH subfamily. Because 3144-AMC was selectively hydrolyzed by FAP, AMC fluorescence remained at baseline in the DPP4, DPP8, DPP9, DPP2, and PREP reactions.*

In vitro pharmacology of AVA6000 vs Doxorubicin

The cell permeability and cytotoxicity of AVA6000 in the absence and presence of FAP in human cell lines was assessed *in vitro*. Following incubation of AVA6000 (20 μM final concentration) with monolayer cultures of HEK-mFAP and HEK-mock cells, intact AVA6000 was found to accumulate in neither HEK-mFAP nor HEK-mock cells over the 24-hour incubation period (figure 3). In contrast, released doxorubicin accumulated in the HEK-mFAP cells in a time-dependent manner, whereas no doxorubicin was detected in HEK-mock cells (figure 3). These data indicate that intact AVA6000 does not readily penetrate the HEK cell, and that cleavage of AVA6000 by cell-surface FAP is required in order for doxorubicin to gain access to intracellular targets.

Cellular permeability of AVA6000 and released doxorubicin +/- FAP

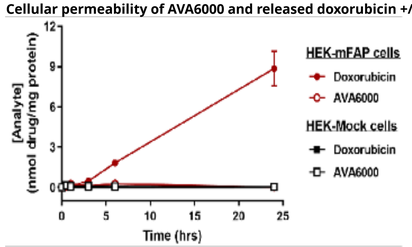
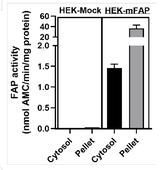


Figure 3: *Monolayer cultures of HEK-mFAP and HEK-mock cells were incubated at 37°C with AVA6000 (20 μM final concentration). After 0, 0.5, 1, 3, 6 and 24 hours, the cells were harvested from triplicate cultures at each time point and washed in PBS containing the FAP inhibitor 5057 (1 μM final concentration), and AVA6000 and released doxorubicin concentrations in cell lysates were analysed by LC-MS/MS. Mean (± SEM) of AVA6000 and doxorubicin concentrations measured at each timepoint are shown on the y-axis.*

A



B

	FAP Activity (nmol AMC/min/mg protein)			
	HEK-Mock		HEK-mFAP	
Fraction	Mean	SEM	Mean	SEM
Cytosol	0.002	0.0004	1.5	0.09
Pellet	-0.001	0.0004	37	6.3

Figure 4: FAP activity associated with membrane (pellet) fraction was approximately 25-fold higher (37 nmol AMC/min/mg protein) in HEK-mFAP cells, than the cytosolic fraction (1.5 nmol AMC/min/mg protein) (Fig 4A). However, FAP activity in both membrane and cytosolic fractions of HEK-mock cells was beneath the level of detection (Fig 4B). FAP activity was measured in triplicate by the 3144-AMC fluorometric assay. The same data are presented in bar chart (A) and table (B).

Tumor cell lines were incubated with AVA6000 +/- human recombinant soluble FAP, or with doxorubicin hydrochloride in order to investigate the cytotoxic activity of AVA6000 before and after hydrolysis by FAP. AVA6000 cytotoxicity was also investigated in cultures of HEK-mFAP and HEK-mock cells. The effect of adding soluble FAP on the cytotoxic activity of AVA6000 was investigated for all the cell lines with the exception of HEK-mFAP. Using cultures containing AVA6000 and FAP inhibitor as baseline controls, addition of AVA6000 and soluble FAP resulted in significant ($P < 0.05$) cytotoxicity in cultures of all 11 tumor cell lines (table 2). Using cultures containing AVA6000 alone as controls, addition of AVA6000 and soluble FAP resulted in increased cytotoxicity in all the cell line cultures. Transfection of HEK cells with mFAP appeared to confer cell-intrinsic sensitivity to AVA6000, as indicated by significantly lower EC_{50} values ($P < 0.0001$) in HEK-mFAP cell cultures incubated with AVA6000 compared with HEK-mock cell cultures incubated with AVA6000 and FAP inhibitor (table 2). For the tumor cell lines tested, with the exception of MCF-7, which was resistant to doxorubicin, intact AVA6000 was approximately 80-fold to 4,000-fold less cytotoxic than doxorubicin *in vitro*. EC_{50} comparisons indicated that the sensitivity of each tumor cell type to FAP-activated AVA6000 corresponded quite closely to its intrinsic sensitivity to doxorubicin *in vitro*.

Table 2: In vitro cell line cytotoxicity by AVA6000 +/- or FAP inhibitor, and by doxorubicin

Cell line	Mean EC_{50} (μM) ± SEM[a]			
	AVA6000 + FAP Inhibitor[b]	AVA6000	AVA6000 + FAP[c]	Doxorubicin
HEK-Mock	350 ± 46	40 ± 13	0.05 ± 0.004	0.09 ± 0.009
HEK-mFAP	220 ± 30	0.55 ± 0.07	-	0.21 ± 0.03
HT29	140 ± 8.3	210 ± 61	0.84 ± 0.06	0.55 ± 0.07
BxPC-3	400 ± 46	250 ± 34	2.40 ± 0.06	3.20 ± 0.09
MCF-7	210 ± 12	160 ± 14	20 ± 0.95	51 ± 14
MDA-MB-231	150 ± 24	160 ± 17	0.71 ± 0.05	0.46 ± 0.06
OVCAR-3	130 ± 9.3	250 ± 31	0.92 ± 013	1.70 ± 0.15
SK-OV-3	63 ± 20	12 ± 2.3	0.24 ± 0.04	0.45 ± 0.04
NCI-H460	26 ± 8.8	1.1 ± 0.17	0.05 ± 0.004	0.05 ± 0.005
A549	84 ± 13	43 ± 11	0.35 ± 0.03	0.30 ± 0.02
AE17	51 ± 17	0.28 ± 0.03	0.03 ± 0.001	0.03 ± 0.002
MC 17-51	55 ± 7.9	1.1 ± 0.13	0.05 ± 0.006	0.06 ± 0.005

a - Mean concentration of AVA6000 or doxorubicin that produced 50% of maximal inhibition of cell growth (EC_{50}),
b - FAP inhibitor (100 μM final concentration)
c - Recombinant soluble human FAP (25 nM final concentration)

Efficacy in an *in vivo* PDX model of Osteosarcoma

This study was conducted to evaluate the non-clinical anti-tumor activity of AVA6000 in Champions TumorGraft® PDX sarcoma model selected due to high FAP mRNA expression. This PDX model was derived from a patient with prior treatment with ifosfamide, doxorubicin, cisplatin and methotrexate prior to tumor collection and did not respond.

Anti-Tumor Activity for human Sarcoma PDX

Group	Agents	IV Dose (mg/kg)	N	Tumor Volume (Day 77)		
				Mean ± SEM (mm^3)	Adjusted p-Value vs. Control*	% TGI
1	Vehicle	0, QWx4	5	1676 ± 184	-	-
2	AVA6000	12, QWx4	4	715 ± 94	0.0076	67
3	Dox	2, QWx4	5	1310 ± 173	0.2873	25

Table 3 **One-way ANOVA followed by Dunnett's multiple comparisons test.*

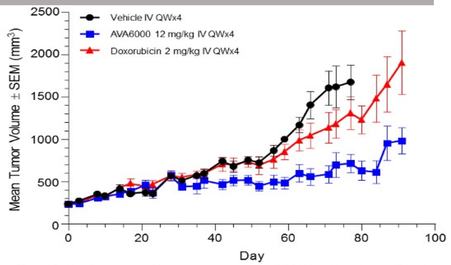


Figure 5: *Heavily pre-treated human sarcoma model, AVA6000 at 12 mg/kg significantly decreased tumor volume while doxorubicin at 2 mg/kg had no significant effect.*

Rat Plasma Pharmacokinetics

Following intravenous administration of AVA6000 at 9 mg/kg to male and female rats, the mean maximum plasma concentration (C_{max}) of AVA6000 was 27800 ng/mL (34035 nM) in males and 20600 ng/mL (25220 nM) in females observed at the first sampling time of 2 minutes post dose. The mean exposure values ($AUC_{(0\text{-}last)}$) in males for AVA6000, 5140 and doxorubicin were 4410 h.ng/mL (5399 h.nM), 978 h.ng/mL (3357 h.nM) and 508 h.ng/mL (935 h.nM), respectively.

Mean plasma PK parameters of AVA6000, doxorubicin and leaving group (5140) in rats

Analyte	Sex	T_{max} (h)	C_{max} (ng/mL)	$T_{1/2}$ (h)	$AUC_{(0\text{-}last)}$ (h.ng/mL)	$AUC_{(0\text{-}\infty)}$ (h.ng/mL)	Vd (mL/kg)	CL (mL/h/kg)
AVA6000	Male	0.033	27800	4.59	4410	4420	13500	2040
	Female	0.033	20600	6.89	2960	2970	30100	3030
5140	Male	0.25	1420	6.25	978	994	81600	9060
	Female	0.25	1220	9.15	753	756	157000	11900
Doxorubicin	Male	0.083	510	31.2	508	710	570000	12700
	Female	0.033	498	33.0	339	491	872000	18300

Table 4: *$AUC_{(0\text{-}last)}$: Area under the concentration versus time curve from the start of dose administration to the time the last quantifiable concentration was observed; $AUC_{(0\text{-}\infty)}$: Area under the concentration versus time curve from the start of dose administration extrapolated to infinite time; CL: Apparent clearance rate; $T_{1/2}$: Apparent terminal elimination half-life; T_{max}: Maximum observed concentration measured after dosing; Vd: Apparent volume of distribution.*

Dog Plasma Pharmacokinetics

Following intravenous administration of AVA6000 at 2 mg/kg, the C_{max} of AVA6000 was 4883 ng/mL (5970 nM), and this was observed at the first sampling time of 2 minutes post dose. The highest mean plasma exposure, according to $AUC_{(0\text{-}last)}$, was for 5140 (662 h.ng/mL; 2273 h.nM), whereas AVA6000 and doxorubicin had $AUC_{(0\text{-}last)}$ of 345 h.ng/mL (422 h.nM) and 87.6 h.ng/mL (161 h.nM), respectively.

Mean plasma PK parameters of AVA6000, doxorubicin and leaving group (5140) in dogs

Analyte	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{(0\text{-}last)}$ (h.ng/mL)	$AUC_{(0\text{-}\infty)}$ (h.ng/mL)	CL (mL/h/kg)	Vd (mL/kg)
AVA6000	4883	0.033	0.458	345	347	6780	4520
5140	458	0.083	ND	662	ND	ND	ND
Doxorubicin	167	0.033	35.5	87.6	123	16700	816000

Table 5. ND: Not determined

Summary

- AVA6000 is a novel and selective FAP activated chemotherapeutic
- In vitro and in vivo data support the tumor microenvironment targeting of AVA6000
- A Phase I trial has been initiated in the UK in a broad spectrum of high FAP expressing tumors
- An Investigational New Drug Application (IND) was in approved in November 2021