**Selectively Targeting Tumor Hypoxia by CP-506, a Next-Generation Hypoxia-Activated Prodrug**

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**Abstract**

Hypoxia is a pervasive feature of human tumors that is acknowledged as a major impediment to treatment success. Hypoxia-activated prodrugs (HAP) are a promising class of antineoplastic agents that can selectively eliminate hypoxic tumor cells. The present study evaluated the hypoxia-selectivity and antitumor activity of CP-506, a DNA alkylating HAP with favorable properties. Radiolytic methods revealed CP-506 to undergo oxygen-reversible one-electron reduction; multi-electron reduction products were readily formed in the absence of oxygen. Cellular metabolism was inhibited completely by oxygen concentrations above 1 µM. CP-506 was activated under anoxia by human diflavin oxidoreductases but was resistant to aerobic activation by human aldo-keto reductase 1C3. CP-506 demonstrated cytotoxicity selectively in hypoxic 2D and 3D cell cultures, with normoxic/anoxic cytotoxicity IC50 ratios up to 203. *In vivo*, the antitumor effects of CP-506 were selective for hypoxic tumor cells and causally related to tumor oxygenation. CP-506 effectively decreased the hypoxic fraction of tumor models and inhibited the growth of a wide range of xenografts, but only when hypoxia was present. A multivariate regression analysis revealed baseline tumor hypoxia and *in vitro* sensitivity to CP-506 significantly correlate with treatment response. Taken together, our results demonstrate the hypoxia-specific cytotoxicity and broad antitumor effects of CP-506.

**In this paper:**

To test whether HF and sensitivity of tumor cell lines to CP-506 defined as the anoxic IC50 value influenced ER or SGD, a database was constructed containing 434 animals (Supplementary Table 4). Only models for which HF and ER were available were included resulting in 381 observations. Outcome data were fit to multiple linear regression with different independent variables: HF (%), anoxic IC50 (μM), absolute cumulative exposure (mg), and mean volume doubling time (VDT, days). Absolute cumulative exposure (further referred to as CP-506 dose) was calculated as the relative dose (mg/kg) times the total number of injections the animal receives times the bodyweight (kg) of the animal at the start of treatment. Since HF could not be determined in the same animal as an outcome variable but was rather determined in sentinel animals in an accompanying experiment, HF was drawn from normal distribution with mean HF and SD estimated for each tumor type in parallel (*vide supra*). Then, HF was randomly assigned to each tumor and a multivariate linear regression analysis was performed (STATA/IC 11.1). This procedure was repeated 500 times. The number of significant associations was counted and the direction of the effect (positive or negative) was recorded for each parameter in the model. The nomogram to predict ER was obtained using R (version 4.0.2).

Code (do file) for simulations is STATA11:

Bootstrapping HF to fit multiple linear regression

. cd C:\01Ala\CP-506\modelling

. do simul

//\*

tempname sim

postfile `sim' p\_dose coef\_dose p\_hf coef\_hf p\_ic50anox coef\_ic50anox p\_vdt coef\_vdt using resout,replace

use datacp506-nomissingER.dta, clear

gen hf\_simul=.

set output error

set seed 17082020

local hf1=17.79

local sd1=6.58

local hf2=16.89

local sd2=4.75

local hf3=5.91 // MDA-MB-231 exp 1

local sd3=0.92 // MDA-MB-231 exp 1

local hf4=18.33

local sd4=3.4 //avarage of all types

local hf5=11.83

local sd5=1.5

local hf8=10.2

local sd8=2.08

local hf9=5.61

local sd9=3.27

local hf10=3.79

local sd10=2.51

local hf11=17.55

local sd11=2.03

local hf12=7.64

local sd12=3.97

local hf13=25.65

local sd13=6.28

local hf14=22.61 // H1650 exp 1

local sd14=3.68 // H1650 exp 1

local hf15=7.97

local sd15=3.66

local hf16=8.43

local sd16=4.18

local hf17=0

local sd17=0

local hf18=0

local sd18=0

local hf19=23.87 // H1650 exp 2

local sd19=5.11 // H1650 exp2

local hf20=6.11 // MDA-MB-231 exp 2

local sd20=1.1 // MDA-MB-231 exp 2

gen p\_dose=.

gen coef\_dose=.

gen p\_hf=.

gen coef\_hf=.

gen p\_ic50anox=.

gen coef\_ic50anox=.

gen p\_vdt=.

gen coef\_vdt=.

forvalues global=1/500 {

replace hf\_sim=`hf1' + `sd1'\* invnorm(uniform()) if tu\_id==1

replace hf\_sim=`hf2' + `sd2'\* invnorm(uniform()) if tu\_id==2

replace hf\_sim=`hf3' + `sd3'\* invnorm(uniform()) if tu\_id==3

replace hf\_sim=`hf4' + `sd4'\* invnorm(uniform()) if tu\_id==4

replace hf\_sim=`hf5' + `sd5'\* invnorm(uniform()) if tu\_id==5

replace hf\_sim=`hf8' + `sd8'\* invnorm(uniform()) if tu\_id==8

replace hf\_sim=`hf9' + `sd9'\* invnorm(uniform()) if tu\_id==9

replace hf\_sim=`hf10' + `sd10'\* invnorm(uniform()) if tu\_id==10

replace hf\_sim=`hf11' + `sd11'\* invnorm(uniform()) if tu\_id==11

replace hf\_sim=`hf12' + `sd12'\* invnorm(uniform()) if tu\_id==12

replace hf\_sim=`hf13' + `sd13'\* invnorm(uniform()) if tu\_id==13

replace hf\_sim=`hf14' + `sd14'\* invnorm(uniform()) if tu\_id==14

replace hf\_sim=`hf15' + `sd15'\* invnorm(uniform()) if tu\_id==15

replace hf\_sim=`hf16' + `sd16'\* invnorm(uniform()) if tu\_id==16

replace hf\_sim=`hf17' + `sd17'\* invnorm(uniform()) if tu\_id==17

replace hf\_sim=`hf18' + `sd18'\* invnorm(uniform()) if tu\_id==18

replace hf\_sim=`hf19' + `sd19'\* invnorm(uniform()) if tu\_id==19

replace hf\_sim=`hf20' + `sd20'\* invnorm(uniform()) if tu\_id==20

replace hf\_sim=0 if hf\_simul<0

regress ER2 abs\_cum\_dose hf\_sim ic50anox vdt

local t = \_b[abs\_cum\_dose]/\_se[abs\_cum\_dose]

replace p\_dose =2\*ttail(e(df\_r),abs(`t'))

replace coef\_dose=\_b[abs\_cum\_dose]

local t = \_b[hf\_sim]/\_se[hf\_sim]

replace p\_hf =2\*ttail(e(df\_r),abs(`t'))

replace coef\_hf=\_b[hf]

local t = \_b[ic50anox]/\_se[ic50anox]

replace p\_ic50anox =2\*ttail(e(df\_r),abs(`t'))

replace coef\_ic50anox=\_b[ic50anox]

local t = \_b[vdt]/\_se[vdt]

replace p\_vdt =2\*ttail(e(df\_r),abs(`t'))

replace coef\_vdt=\_b[vdt]

post `sim' (p\_dose) (coef\_dose) (p\_hf) (coef\_hf) (p\_ic50anox) (coef\_ic50anox) (p\_vdt) (coef\_vdt)

}

postclose `sim'

\*//

. tempname sim

. postfile `sim' p\_dose coef\_dose p\_hf coef\_hf p\_ic50anox coef\_ic50anox p\_vdt coef\_vdt using resout,replace

. use datacp506-nomissingER.dta, clear

. gen hf\_simul=.

(400 missing values generated)

. set output error

end of do-file

. use resout, clear

. save results\_bs, replace

file results\_bs.dta saved