SUPPORTING INFORMATION

XANES spectroscopy to resolve the *in vivo* chemistry of the redox-active KP1019

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SAMPLE PREPARATION AND ANALYSIS

BUFFER PREPARATION

The citrate saline buffer (CS buffer) was made in the following way: To 400 ml of a sterile normal saline (0.9 % w/v NaCl, Sigma Aldrich, CAS 7647-14-5, ≥99.5 %) solution 0.42 g of citric acid anhydrous (Sigma Aldrich, 77-92-9, ≥99.5 %) were added and dissolved completely (solution A). In 400 ml of a sterile normal saline (0.9 % w/v NaCl) solution 0.59 g of sodium citrate dihydrate (Sigma Aldrich, CAS 6132-04-3, ≥99 %) were dissolved (solution B). 156 ml of solution A were combined with 44 ml of solution B. The resulting CS buffer was adjusted to pH 3.5. The carbonate buffer was prepared by dissolving 25 mM of sodium bicarbonate (NaHCO₃, Sigma Aldrich, CAS 144-55-8, ≥99 %), 4 mM of disodium hydrogenphosphate dihydrate (Na₂HPO₄, Sigma Aldrich, CAS 10028-24-7, ≥99.5 %) and 100 mM of sodium chloride (NaCl, Sigma Aldrich, CAS 7647-14-5, ≥99.5 %) in distilled water. The pH was adjusted to pH 7.4 with sodium hydroxide (NaOH, Sigma Aldrich, CAS 1310-73-2, ≥97.0 %).

APPLICATION SCHEMES ANIMAL EXPERIMENTS

At the Slovak Academy of Science BALB/c mice bearing the subcutaneous sarcoma 180 (S180) were treated with 1 (KP1019) and nude mice (bearing no tumors) were treated with 2 (KP1339). 1 was dissolved in aqua ad injectionem, and 2 was dissolved in 5 mM sterile filtered CS buffer prepared as described in the paragraph before. 1 was applied intravenously with dosages of 7.5 mg/kg (mouse B2) and 15 mg/kg (mouse C1) five times 1, 4, 8, 11 and 16 days after tumors had grown to a size of ca. 10 × 10 mm. 2 was applied with a dosage of 40 mg/kg (B1 and F1 mice) one time. The samples of 1 (B2/C1-tumor and B2/C1-liver) were collected one day after the last drug administration. The samples of 2 (B1/F1-liver only) were collected 1 h (mouse B1) and 24 h (mouse F1) after the last drug administration. At the Medical University of
Vienna CB-17 scid/scid (SCID) mice bearing the subcutaneous tumor SW480 (1 × 10^6 cells injected subcutaneously into the right flank) were treated on day 21 with 2. The drug was applied intravenously with a dosage of 40 mg/kg one time. The liver and tumor samples were collected 24 h after the drug administration (mouse 148-1). An overview is given in Table S1.

**XAS AND micro-XRF EXPERIMENTAL SETUPS**

At beamline BM26A (ESRF, Grenoble; France) three low noise ion chambers from Oxford Instruments were used for measurements in transmission mode. The absolute energy calibration was performed using a ruthenium powder (Sigma Aldrich, CAS 7440-18-8, 99.9 %) BN preparation optimized for an absorption edge jump of 1 abs, measured at the same time between ionization chambers two and three. The edge position was determined over the first maximum in the first derivative and used for the energy calibration. In fluorescence mode a 9-element monolithic Ge detector with a maximum count rate per element around 150 kHz and an energy resolution < 250 eV at 5.9 keV was used. An Oxford CCC 1204 cryostat provided a sample environment of 20 K. The ESRF storage ring was operated at 6 GeV in the 7/8+1 filling mode. The beamline BM26A was equipped with a double crystal Si(111) monochromator and a bending magnet source giving an energy range of 5−30 keV (flux of 1 × 10^{11} ph/s). Higher harmonics were rejected using two mirrors with Pt and Si coatings.

The XANES signals were measured at the Ru K-edge with a pre-edge region from 21869 eV to 22062 eV with a step size of 10 eV, an edge region from 22083 eV to 22150 eV with a step size of 1.3 eV. The scanning times were set to 2 sec in the pre-edge and 10 sec in the edge region. The k-space was measured from 3 to 10 Å⁻¹ with a step size of 0.05 Å⁻¹. The post edge scanning time was set to 5−25 sec, increasing according to a predefined curve.

The EXAFS modulations were measured at the Ru K-edge with a pre-edge region from 21869 eV to 22083 eV with a step size of 10 eV, an edge region from 22099 eV to 22161 eV with a step
size of 1.3 eV. The k-space was measured from 3 to 14 Å\(^{-1}\) with a step size of 0.05 Å\(^{-1}\). The scanning times were 1 sec in the pre-edge, 5 sec in the edge and 5 – 25 sec (22161 eV – 22917 eV), increasing according to a predefined curve, in the post-edge region.

On the tissue samples 40 to 80 scans per sample were collected. The spectra of the model compounds are the average of 2 scans and 4 to 10 scans were collected on the liquid samples.

At the FLUO beamline (ANKA, Karlsruhe, Germany) the samples were scanned with a X-ray beam focused to a spot size of 10 µm using a poly-capillary. The fluorescence signals were detected with a KETEK AXAS-M Si drift detector with a counting time of 10 sec per spot. The incoming beam intensity was measured with an ionization chamber. The 10 µm thick thin sections were positioned in a 45° angle to the incoming beam and the detector.

**XAS AND micro-XRF ANALYSIS**

**XANES ANALYSIS**

The pre-edge background was removed by a linear approximation in the range of −30 eV to −250 eV before the edge. This baseline was subtracted from the entire spectrum. The normalization of the model compounds was accomplished fitting a quadratic polynomial in the post edge region from 200 eV to 750 eV. The tissue and drug solution samples were normalized approximating a straight line in the range of 80 eV to 200 eV post the edge. The fitted functions were extrapolated to the first absorption maximum and the absorbance was set to unity there. The edge position was determined as the maximum in the first derivative of the spectrum (inflection point of the steeply rising edge).

**EXAFS ANALYSIS**

*FEFF analysis:* The pre-edge background subtraction and the normalization were performed as described for the XANES spectra. The normalized EXAFS signals were extracted using the AUTOBK algorithm incorporated into IFEFFIT. The background function is constructed using
cubic splines with evenly spaced knots throughout the data region. The number of splines is defined as \((2R_{\text{bkg}} \Delta k)/\pi\). Where the \(R_{\text{bkg}}\) value defines the frequencies which are allowed to be removed by the smoothly varying background spline, and is usually set to one half of the first shell distance (longer frequencies will be removed). In our case \(R_{\text{bkg}}\) was set to 1 Å and the spline range \(\Delta k\) to 0.5 – 14.4 Å\(^{-1}\). The Fourier transform was constructed using a Hanning window from 3 – 13 Å\(^{-1}\) and a \(\delta k\) of 1 Å\(^{-1}\). The back-Fourier transform was extracted using a Hanning window including the first shell peak and a \(\delta R\) of 0 Å.

FEFF7 was used for the calculation of the theoretical EXAFS amplitudes and phases. The program package ARTEMIS was used to apply the EXAFS equation, shown in equation (3), for the fitting of the \textit{ab initio} amplitude and phases to the experimental data.

\[
\chi(k) = S_0^2 \sum_j \sum_{i=1}^{N_j} f_{ij}(\pi, k, R_j) e^{-2\sigma_j^2 k^2} e^{-2R_j/\lambda_j(k)} \sin[2kR_j + \phi_j(k)]
\]  

(3)

The neighboring atoms or scatterers are divided in shells \(j\) consisting of atoms of the same atomic number and distance from the absorber atom. \(N_j\) defines the number of atoms in each shell \(j\) at the distance \(R_j\) from the absorber atom. The \(f_{ij}(\pi, k, R_j)\) term is the \textit{ab initio} calculated amplitude function for shell \(j\), and \(exp[-2\sigma_j^2 k^2]\) accounts for the damping due to static disorder and thermal motion in the absorber scatterer distance (Debye-Waller factor). The mean free path term \(exp[-2R_j/\lambda_j(k)]\) considers losses through inelastic scattering, where \(\lambda_j(k)\) is the electron mean free path. The term \(sin[2kR_j + \phi_j(k)]\) reflects the EXAFS oscillations of the spectrum and \(\phi_j(k)\) is the \textit{ab initio} phase function for the \(j^{th}\) shell. The amplitude reduction factor \(S_0^2\) accounts for shake-up/shake-off events at the central atom.

The shift in the edge position \((E_0)\) and the Debye-Waller factor \((\sigma^2)\) were refined for all first shell scatterers. The absorber – scatterer distance \((R_j)\) was refined over a contraction expansion factor \(\alpha\) (alpha) relative to the starting value. The amplitude reduction factor \(S_0^2\) was set to 1.0
throughout the fits. The number of scatterers $N_j$ was fixed to the one known from the crystallographic data. All fits were performed on the backtransformed, Fourier filtered spectra.

**DL-EXCURV analysis:** The $k^3$ weighted EXAFS signals were extracted using the program PySpline. The edge energy was set to the values as determined in the XANES analysis. The pre- and post-edge backgrounds were subtracted fitting a straight line and a three segment spline of third order polynomials, respectively. The Fourier transformation was conducted with a Kaiser-Bessel window with a $\delta k = 0.1$ Å$^{-1}$. DL-EXCURV calculated the theoretical EXAFS spectra for the defined structural model based on the curved wave method for single scattering. The ground- and excited-state exchange contributions to the effective potential were calculated with the von Barth method. The structural parameters atomic distance ($R$), Debye–Waller factor ($2\times\sigma^2$) and the residual shift (EF) of the edge energy ($E_0$) were refined to obtain the best fit between experiment and theory. The amplitude reduction factor (AFAC) and the number of scatterers (N) were fixed. The quality of the fit was indicated by the R-factor. The number of free parameters ($N_F$) was always inspected to be less than the number of independent data points ($N_{IND}$). The fits were performed on the raw $k^3$ weighted EXAFS data.

**STRUCTURAL MODEL**

The minimal distinguishable distances are determined according to the formula $\delta R = \pi/(2\times k_{max})$, where $k_{max}$ represents the $k$-range used for the Fourier transformation. For all model compounds $\delta R = 0.157$ Å and is much lower than the mean distance differences of O/N and Cl/S shells of about 0.300 Å. This makes it possible to distinguish these first shell atom species and perform sub shell fits for O/N and Cl/S and determine their mean distance to the central Ru atom.

**PCA AND LSF**

The program Sixpack was used to perform the principal component analysis (PCA), leastsquare fits (LSF) and linear combination analysis (LCA). A PCA algorithm based on single
value decomposition (SVD) and a target transformation were used as described in Ressler et al. The normalized XANES spectra of the tumor and liver samples were taken for the analysis in the energy range of 22100 eV to 22200 eV. The normalization was performed as described before in the XANES section. Target transformation on the vector subspace defined by the first principal component was done with the known reference spectra. LSF was conducted based on the Levenberg–Marquardt algorithm.

micro-XRF ANALYSIS

The micro-XRF maps were analyzed with the program package PyMCA. The detected fluorescence signals \(I_f\) for a given element were normalized with the incoming beam intensity \(I_0\). The resulting micro-XRF maps were logarithmically scaled to pronounce hot spots in the maps and the upper cut off was set to the 99 percentile of the measured intensities.
TABLES

Table S1: Application schemes and dosages of 1 and 2 in the animal tests.

| sample       | drug | dosage | tumor   | mouse     | application | sacrificed |
|--------------|------|--------|---------|-----------|-------------|------------|
| C1-tumor     | 1    | 15 mg/kg | S180    | BALB/c    | 5 times     | 24 h       |
| C1-liver     | 1    | 15 mg/kg | ---     | BALB/c    | 5 times     | 24 h       |
| B2-tumor     | 1    | 7.5 mg/kg | S180    | BALB/c    | 5 times     | 24 h       |
| B2-liver     | 1    | 7.5 mg/kg | ---     | BALB/c    | 5 times     | 24 h       |
| 148-1-tumor  | 2    | 40 mg/kg | SW48    | SCID      | 1 time      | 24 h       |
| 148-1-liver  | 2    | 40 mg/kg | ---     | SCID      | 1 time      | 24 h       |
| B1-liver     | 2    | 40 mg/kg | ---     | nude mouse| 1 time      | 1 h        |
| F1-liver     | 2    | 40 mg/kg | ---     | nude mouse| 1 time      | 24 h       |

The mice were sacrificed after 24 or 1 h after the last drug administration.
Table S2: 1st shell fits of the model compounds using theoretical amplitudes and phases provided by the FEFF code.

| comp. path | Nfix | R [Å] | Rcryst [Å] | ΔR [Å] | σ² [Å² 10⁻³] | E₀ [eV] | fit index [%] | cryst. ref. |
|------------|------|-------|------------|--------|---------------|--------|--------------|------------|
| Ru−O       | 6    | 2.02(2) | 2.006 | 0.014 | 2.29±1.03 | 2.5±2.8 | 2.7 | [53,54] |
| Ru−N       | 6    | 2.12(2) | 2.093 | 0.027 | 2.42±0.63 | 4.5±1.5 | 1.0 | [55] |
| Ru−N/O     | 2/1  | 2.08(2) | 2.065 | 0.015 | 1.52±0.71 | 4.9±1.7 | 1.5 | [56] |
| Ru−Cl      | 3    | 2.35(2) | 2.337 | 0.013 |              |        |        |            |
| Ru−N       | 4    | 2.09(2) | 2.071 | 0.019 | 2.57±1.05 | 6.1±2.4 | 2.0 | [27] |
| Ru−Cl      | 2    | 2.35(2) | 2.331 | 0.019 |              |        |        |            |
| Ru−N/C     | 4/4  | 3.07(3) | 3.064 | 0.006 |              |        |        |            |
| Ru−N       | 3    | 2.08(1) | 2.068 | 0.012 | 1.24±0.83 | 3.8±2.0 | 2.4 | [57] |
| Ru−Cl      | 3    | 2.36(2) | 2.343 | 0.017 |              |        |        |            |
| Ru−N       | 2    | 2.06(1) | 2.062 | −0.002 | 1.42±0.59 | 2.0±1.6 | 2.1 | [9-11] |
| Ru−Cl      | 4    | 2.37(1) | 2.364 | 0.006 |              |        |        |            |
| Ru−S/Cl    | 1/3  | 2.364(7) | 2.369 | −0.005 |              |        |        |            |
| Ru−N       | 4    | 2.08(2) | 2.062 | 0.018 | 2.06±0.56 | 3.3±1.9 | 2.5 | [27] |
| Ru−Cl      | 2    | 2.43(2) | 2.418 | 0.012 |              |        |        |            |
| Ru−N/C     | 4/4  | 3.07(3) | 3.059 | 0.011 | 4.73±2.78 |        |        |            |
| Ru−S/Cl    | 3/3  | 2.379(4) | 2.369 | 0.010 | 2.04±0.23 | 4.9±0.7 | 0.5 | [60] |
| Ru−N       | 2    | 2.080(4) | 2.076 | 0.004 | 2.98±0.93 | 4.1±1.9 | 2.5 | [59] |
| Ru−S/Cl    | 2/2  | 2.403(4) | 2.400 | 0.003 |              |        |        |            |

Nfix is the fixed coordination number, R is the average distance, Rcryst is the crystallographic value, ΔR is the difference between R and Rcryst, σ² is the Debye Waller factor, E₀ is the residual shift of the edge energy.
Table S3: DL–EXCURV EXAFS fits of the model compounds.

| comp. path | N<sub>fix</sub> | N<sub>path</sub> | R<sub>cryst</sub> [Å] | R<sub>fit</sub> [Å] | ∆R [Å] | σ<sup>2</sup> [Å<sup>2</sup> 10<sup>-3</sup>] | E<sub>0</sub> [eV] | R<sub>fit</sub> [%] | fit index | cryst. ref. |
|------------|----------------|-----------------|----------------|----------------|--------|----------------|---------|-------------|-----------|-------------|
| Ru–O       | 6              | 2.015(6)        | 2.006          | 0.009          | 2.72±0.50 | −1.5±1.0       | 36.1    | 1.04        | [53,54]   |
| Ru–C       | 6              | 2.94(2)         | 2.913          | 0.027          | 1.69±1.42 |
| Ru–N       | 6              | 2.107(3)        | 2.079          | 0.028          | 2.31±0.26 | −2.9±0.6       | 22.4    | 0.37        | [55]      |
| Ru–N/O     | 2/1            | 2.062(9)        | 2.065          | −0.003         | 2.35±0.88 | −4.0±0.8       | 25.4    | 0.54        | [56]      |
| Ru–Cl      | 3              | 2.340(4)        | 2.337          | 0.003          | 1.72±0.33 |
| Ru–N/C     | 4              | 3.07(2)         | 3.011          | 0.059          | 4.47±2.57 |
| Ru–N       | 4              | 2.059(7)        | 2.071          | −0.012         | 1.82±0.64 | −2.5±0.8       | 34.9    | 0.97        | [27]      |
| Ru–Cl      | 2              | 2.330(7)        | 2.331          | −0.001         | 2.74±0.64 |
| Ru–N/C     | 4/4            | 3.07(1)         | 3.059          | 0.011          | 3.16±1.01 |
| Ru–N       | 3              | 2.085(9)        | 2.068          | 0.017          | 2.09±0.92 | −4.8±0.8       | 24.4    | 0.53        | [57]      |
| Ru–N/C     | 3/3            | 3.09(1)         | 3.060          | 0.030          | 4.05±1.56 |
| Ru–N       | 2              | 2.10(2)         | 2.062          | 0.032          | 3.62±1.72 | −6.0±0.8       | 20.5    | 0.31        | [9-11]    |
| Ru–Cl      | 4              | 2.374(4)        | 2.364          | 0.010          | 1.83±0.24 |
| Ru–N/C     | 2/2            | 3.11(3)         | 3.051          | 0.060          | 6.04±3.11 |
| Ru–N       | 6              | 2.107(3)        | 2.093          | 0.014          | 2.31±0.26 | −2.9±0.6       | 32.5    | 0.76        | [58]      |
| Ru–Cl      | 2              | 2.07(1)         | 2.074          | −0.004         | 1.65±1.04 | −5.4±0.8       | 19.3    | 0.32        | [59]      |
| Ru–N/C     | 1/3            | 2.360(4)        | 2.369          | −0.009         | 2.15±0.24 |
| Ru–N/C     | 2/2            | 3.10(2)         | 3.060          | 0.040          | 3.83±2.00 |
| Ru–N       | 4              | 2.062(6)        | 2.064          | −0.002         | 1.97±0.61 | −3.9±0.7       | 38.9    | 1.06        | [27]      |
| Ru–Cl      | 2              | 2.431(7)        | 2.412          | 0.019          | 2.77±0.64 |
| Ru–N/C     | 4/4            | 3.07(2)         | 3.059          | 0.011          | 6.54±1.99 |
| Ru–Cl/S    | 3/3            | 2.377(2)        | 2.369          | 0.008          | 2.59±0.15 | −5.6±0.5       | 15.4    | 0.17        | [60]      |
| Ru–C       | 2              | 2.07(1)         | 2.076          | −0.005         | 1.84±0.72 | −5.5±0.7       | 20.5    | 0.36        | [59]      |
| Ru–S/Cl    | 2/2            | 2.400(4)        | 2.399          | 0.001          | 4.63±0.29 |
| Ru–N/C     | 2/2            | 3.10(1)         | 3.073          | 0.027          | 4.32±1.84 |
| Ru–C       | 4              | 3.39(2)         | 3.439          | −0.049         | 3.89±2.35 |

N<sub>fix</sub> is the fixed coordination number, R is the average distance, R<sub>cryst</sub> is the crystallographic value, ∆R is the difference between R and R<sub>cryst</sub>, σ<sup>2</sup> is the Debye Waller factor, E<sub>0</sub> is the residual shift of the edge energy, R<sub>fit</sub> is the quality of the fit, fit index is the sum of the square of the residuals.
Table S4: Least square fits of 4, 3 and 11 and combinations of them to 1 in BN, 4 h in CS buffer, 5 h in CS buffer in the presence of GSH and 30 min in carbonate buffer in the presence of apoTf.

| Sample                  | 1  | 3   | 4   | 11  | 4   |
|-------------------------|----|-----|-----|-----|-----|
|                         | N  | O   | Cl/S| oxidation state |
| 1 in BN                 |    |     |     |     |     |
| $\chi^2$                | 8.339 | 4.261 | 1.609 | 0.885 |
| $\chi^2_{\text{red}}$   | 0.00417 | 0.00213 | 0.00081 | 0.00044 |
| $\Delta E$              | 2.07 | 1.52 | −1.21 | −0.23 |
| $\Sigma_C$             | 0.970(2) | 0.976(1) | 1.000(7) | 0.9934 |
| Ru$^{3+}$N$_6$          |    |     |     |     | 0.29(1) |
| Ru$^{3+}$O$_6$          | --- | --- | --- | --- | 0.02(1) |
| Ru$^{3+}$Cl$_3$S$_3$    | --- |     |     |     | 0.670(7) |
| 1 in CS buffer 4 h      |    |     |     |     |     |
| $\chi^2$                | 6.602 | 2.777 | 2.162 | 0.466 |
| $\chi^2_{\text{red}}$   | 0.00330 | 0.00140 | 0.00108 | 0.00023 |
| $\Delta E$              | 0.78 | 0.17 | −2.47 | −1.1 |
| $\Sigma_C$             | 0.962(1) | 0.970(1) | 0.9923(8) | 0.9825 |
| Ru$^{3+}$N$_6$          |    |     |     |     | 0.436(9) |
| Ru$^{3+}$O$_6$          | --- | --- | --- | --- | 0.031(7) |
| Ru$^{3+}$Cl$_3$S$_3$    | --- |     |     |     | 0.515(5) |
| 1 with GSH in CS buffer 5 h |    |     |     |     |     |
| $\chi^2$                | 11.524 | 6.447 | 2.296 | 1.227 |
| $\chi^2_{\text{red}}$   | 0.00576 | 0.00323 | 0.00115 | 0.00061 |
| $\Delta E$              | 2.37 | 2.02 | −0.21 | 0.64 |
| $\Sigma_C$             | 1.000(1) | 0.974(2) | 0.980(1) | 0.9946 |
| Ru$^{3+}$N$_6$          |    |     |     |     | 0.28(1) |
| Ru$^{3+}$O$_6$          | --- | --- | --- | --- | 0.03(1) |
| Ru$^{3+}$Cl$_3$S$_3$    | --- |     |     |     | 0.678(7) |
| 1 with apoTf in carb. buffer 30 min |    |     |     |     |     |
| $\chi^2$                | 5.650 | 2.778 | 0.961 | 10.601 | 0.589 |
| $\chi^2_{\text{red}}$   | 0.00139 | 0.00048 | 0.00530 | 0.00030 |
| $\Delta E$              | −0.12 | −0.87 | −2.72 | −0.80 |
| $\Sigma_C$             | 1.0021(9) | 1.0093(6) | 1.025(2) | 1.0095 |
| Ru$^{3+}$N$_6$          |    |     |     |     | 0.65(1) |
| Ru$^{3+}$O$_6$          | --- | --- | --- | --- | 0.277(8) |
| Ru$^{3+}$Cl$_3$S$_3$    | --- |     |     |     | 0.083(5) |
$\chi^2$ is the goodness of fit parameter scaled to the estimated uncertainty, red. $\chi^2_{\text{red}}$ is $\chi^2$ divided by the number of free parameters, $\Delta E$ accounts for the energy shift of the sample data, $\Sigma C$ is the sum of the fitted components and is the scaling factor applied to the fitted spectrum.
CHARTS AND FIGURES

Figure S1: Target transformation of the reference compounds 1, 5, 6 and 7 into the vector subspace spanned by the first principal component. Spectra are plotted with an arbitrary vertical shift.

Figure S2: Fit and corresponding residuals of 5 to the tissue samples B2-liver (left) and B2-tumor (right) derived from mice treated with 1.