Modulating the behaviour of pancreatic tumour cells

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Chapter 4: Iridium Complex with Antiangiogenic Properties

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Abstract

Substitutionally inert metal complexes are promising emerging scaffolds for targeting enzyme active sites. (Meggers E., 2007) Our group has demonstrated over the last several years that inert ruthenium (II) complexes can serve as highly selective nanomolar and even picomolar inhibitors of protein kinases. (Meggers E., et al., 2007) Octahedral metal coordination geometries in particular offer new gateways to design rigid, globular molecules with defined shapes that can fill protein pockets such as enzyme active sites in a unique fashion (Figure 1) (Maksimoska J., et al., 2008). However, the large number of possible stereoisomers does not only provide new structural opportunities (e.g. the complex illustrated in Figure 1 can form up to 24 stereoisomers if the ligands A-D differ), but also poses a formidable challenge due to the limited ability to control stereochemistry in the course of ligand exchange reactions (Coe B. J. and Glenwright, S. J., 2000). A continued progress in this area of inorganic medicinal chemistry therefore requires the development of strategies for the stereo-controlled synthesis of octahedral metal complexes. Although most of our previous efforts were focused on ruthenium (II) complexes, we envisioned that octahedral iridium(III) (Ir(III)) complexes might be attractive scaffolds for two reasons: First, coordinative bonds with Ir(III) tend to be very inert (J. Burgess, 1972) and therefore Ir(III) complexes should be able to serve as stable scaffolds for the design of enzyme inhibitors (Kwon T.H. et al., 2008; Yu M. et al., 2008; K. K. W. Lo et al., 2008). Second, octahedral Ir(III) complexes can be accessed from square planar Ir(I) complexes by stereospecific oxidative addition reactions (Mondal J. U. and Blake D. M., 1982. This provides a powerful tool to control the stereochemistry of octahedral Ir(III) complexes. In this study, we present the discovery of a bioactive octahedral iridium (III) complex, synthesized through oxidative addition as the key synthetic step. The organometallic compound functions as a nanomolar and selective inhibitor of the protein kinase Flt4 (Fms-related tyrosine kinase 4), also known as VEGFR3 (vascular endothelial growth factor receptor 3) (Karkkainen M. J. and Petrova T., 2000). Flt4 is involved in angiogenesis and lymphangiogenesis (Karkkainen M. J. and Petrova T., 2000) and we demonstrate that this nontoxic organoiridium compound can indeed interfere with the development of blood vessels *in vivo* in two different zebrafish angiogenesis models.

Experimental procedures and results

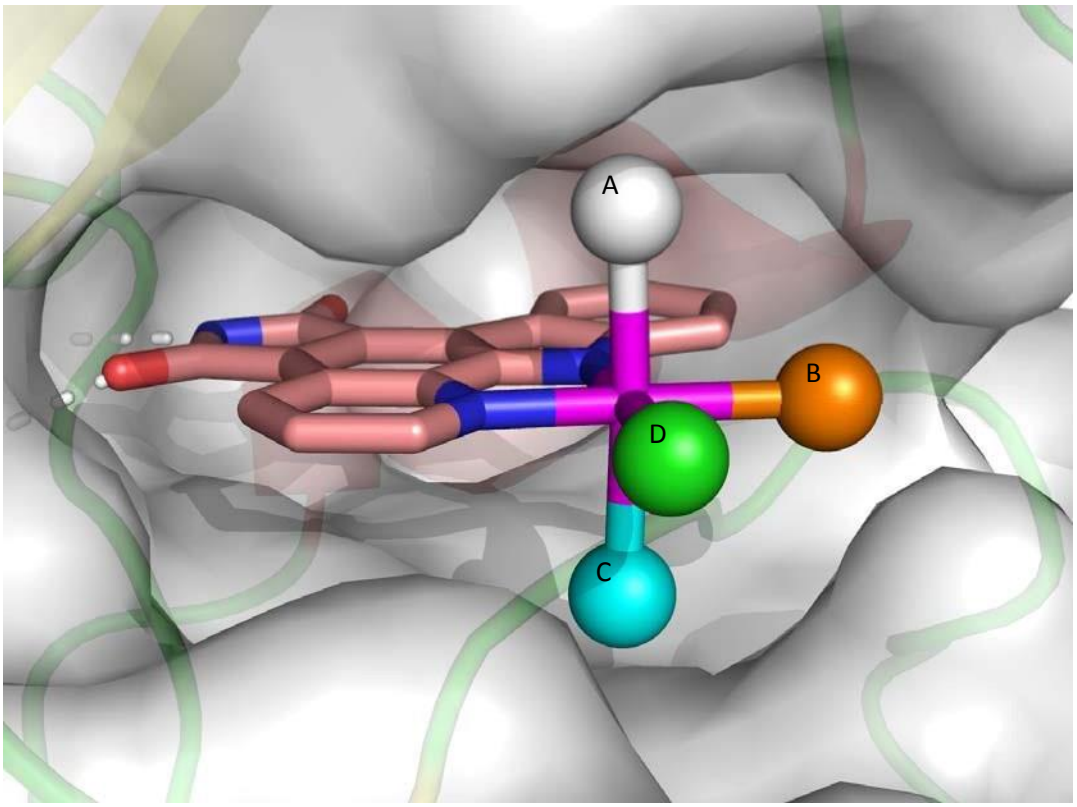
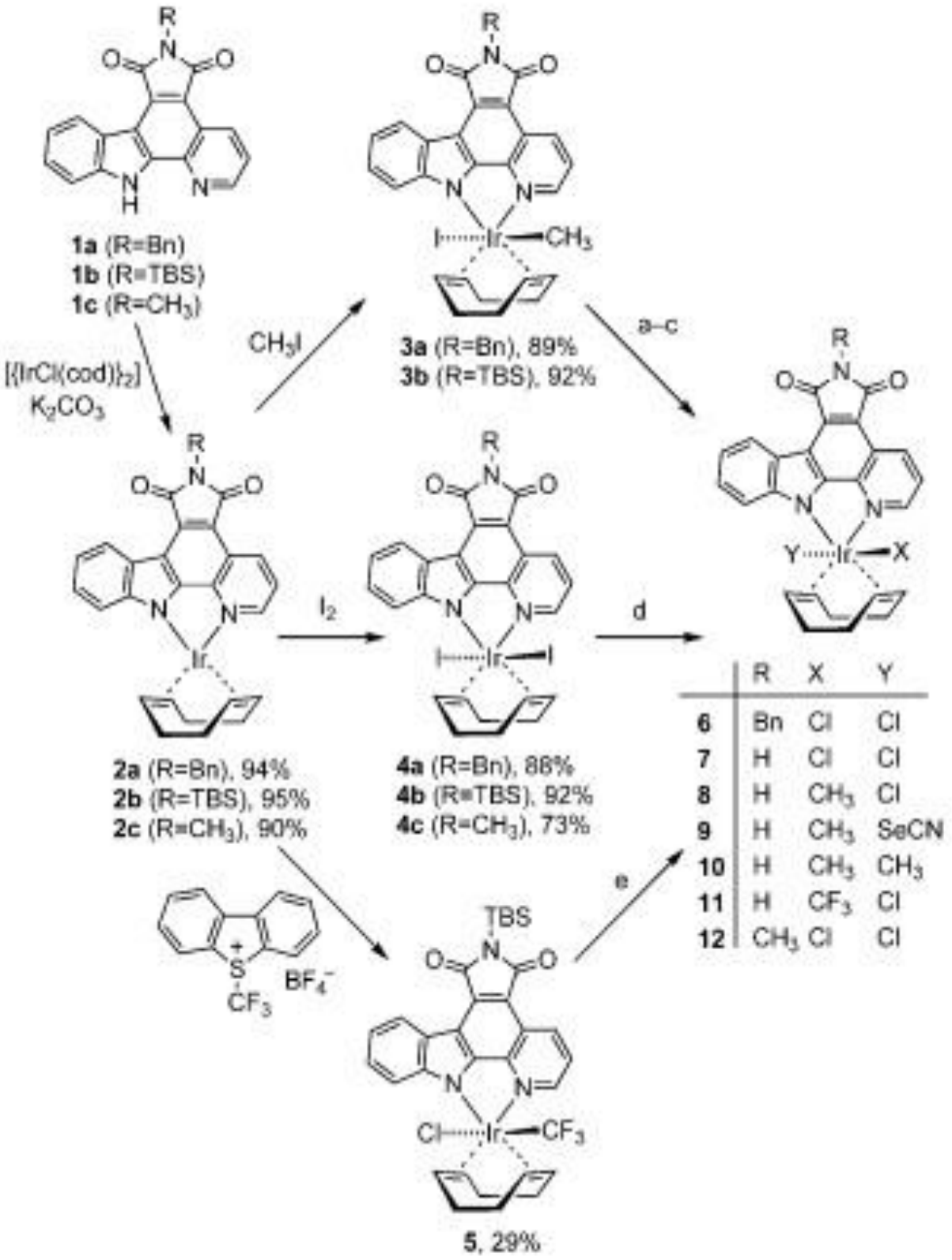


Figure 1. Illustration of an octahedral pyridocarbazole metal complex bound to the active site of a protein kinase. The coordinating ligands A-D are capable of controlling kinase affinities and selectivities, if arranged properly.

We started with iridium complexes containing our recently developed pyridocarbazole pharmacophore bidentate ligand that targets the compounds to the ATP-binding site of protein kinases (Figure 1). For initial synthetic studies we used the benzyl protected ligand **1a** and reacted it with $[IrCl(COD)]_2$ (COD = 1,5cyclooctadiene) in MeCN/MeOH (2:1) in the presence of K_2CO_3 to afford the iridium (I) complex **2a** in 94% (Scheme 1). This slightly air sensitive complex efficiently undergoes oxidative addition. For example, the reaction of **2a** with freshly distilled CH_3I in the dark provided the stable octahedral complex **3a** (89%) stereospecifically as the *trans* oxidative addition product (G. Mestroni, 1974). Similarly, the reaction of **2a** with I_2 afforded the octahedral complex **4a** in 88%. A crystal structure of complex **4a** is shown in Figure 2 and reveals the *trans* coordination of iodine.

Importantly, the iodide ligands in **4a** can further be subjected to substitution chemistry, as exemplified by the conversion to the dichloride complex **6** upon treatment with tetrabutylammonium chloride (TBAC) (88%).

Encouraged by these results, we synthesized in an analogous fashion, starting with the TBS (Tris buffered saline)-protected pyridocarbazole **1b**, a small library of iridium complexes **7**-**11** bearing unprotected maleimide moieties essential for undergoing hydrogen bonds with the hinge region of the ATP- binding site of protein kinases (Scheme 1 and Supplementary Information).


1a (R=Bn)
1b (R=TBS)
1c (R=CH3)
[{IrCl(cod)}2]
K2CO3
CH3I
3a (R=Bn), 89%
3b (R=TBS), 92%
a–c
2a (R=Bn), 94%
2b (R=TBS), 95%
2c (R=CH3), 90%
I2
4a (R=Bn), 86%
4b (R=TBS), 92%
4c (R=CH3), 73%
d
BF4−
e
5, 29%
R X Y
6 Bn Cl Cl
7 H Cl Cl
8 H CH3 Cl
9 H CH3 SeCN
10 H CH3 CH3
11 H CF3 Cl
12 CH3 Cl Cl

Scheme 1**.**Synthesis of octahedral iridium complexes through oxidative addition as the key step. Reaction conditions a-c: a) TBAC, THF, 79% of **8**; b) KSeCN, DMF, 83% of **9**; c) MeMgBr, CuI, THF, 63%; TBAF, CH_2Cl_2, 73% of **10**; d) TBAC, THF, 88% of **6**, 59% of **7**, and 71% of **12**; e) TBAC, THF, microwave (70 °C, 17 min, 20 watts), 50% of **11**. TBAC = tetrabutylammonium chloride.

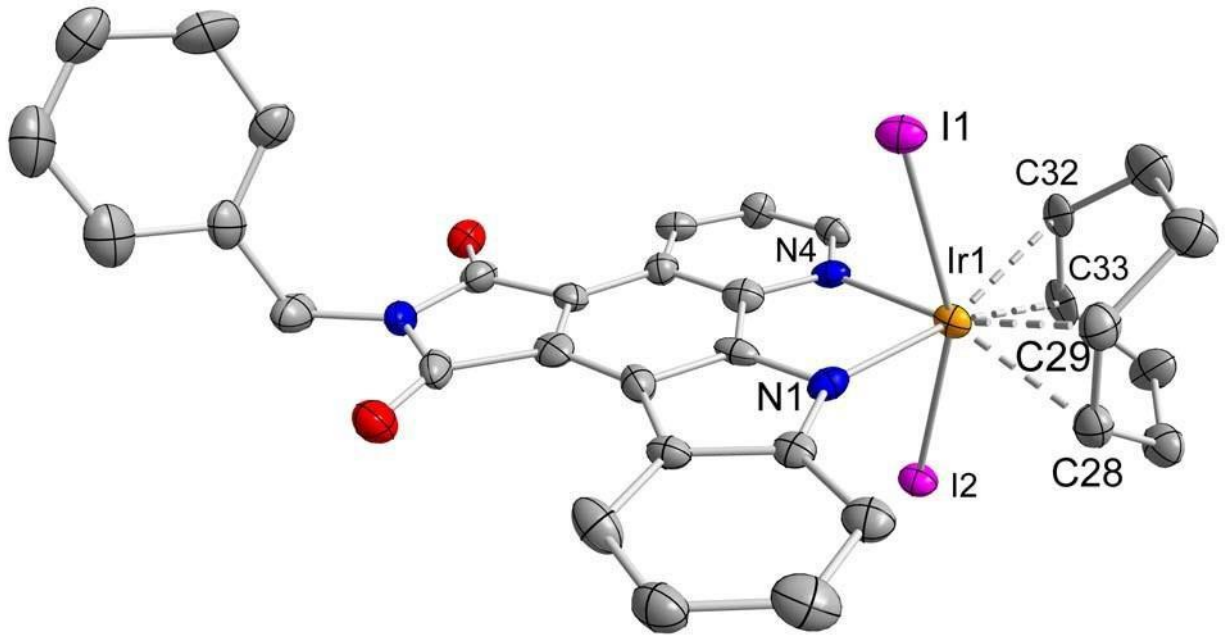


Figure 2. ORTEP representation (50% thermal ellipsoids) of the crystal structure of complex **4a**. Selected bond distances: N1-Ir1. 2.070(6), N4-Ir1 2.122(6), C28-Ir1 2.240(8), C29-Ir1 2.251(7), C32-Ir1 2.222(7), C33-Ir1 2.252(7), I1-Ir1 2.733(6), I2-Ir1. 2.714(6) Å. Selected bond angles: N1-Ir1-I2 82.29(16), N4-Ir1-I2 81.44(16), N1-Ir1-I1 80.42(16), N4-Ir1-I1 81.15(16), I2-Ir1-I1 157.12(2)°.

In order to quickly evaluate the kinase inhibition properties of this class of iridium complexes, we selected compound **8** as a representative member and screened it against a panel of protein kinases. As a result, at a concentration of 1 µM **8** and 10 µM ATP only 12 out of 263 kinases showed activities below 25% (see supporting information). Intriguingly, the receptor tyrosine kinase Flt4 (Fms-related tyrosine kinase 4), which was not selectively inhibited by any of the previously reported ruthenium scaffolds, (Meggers E., 2007; Meggers E., et al., 2007) displayed under these conditions only an activity of 2%. We therefore selected Flt4 for further studies and tested the small library of iridium

compounds **7**-**11** against this kinase by determining the concentrations of **7**-**11** at which Flt4 kinase activity was reduced to 50% (IC_{50}) at an ATP concentration of 100 µM. As expected, ligands X and Y of compounds **7**-**11** (Scheme 1) turned out to have a profound effect on the inhibition properties. Whereas compound **8** displayed an IC_{50} value of 211±23 nM, the more hydrophobic dimethyl derivative **10** had a significantly lower potency (IC_{50} = 1007±129 nM). Replacing the CH_3 group in **8** against CF_3 also reduced affinity with an IC_{50} value of 369±68 nM for **11**. Furthermore, as seen for compound **9**, substituting the chloride for selenocyanate decreased the inhibition potency slightly compared to complex **8** (IC_{50} = 326±52 nM for complex **9**). However, complex **7** with X = Y = Cl showed a significantly improved activity with an IC_{50} value of 123±14 nM, thus being almost an order of magnitude more potent against Flt4 than the related dimethyl derivative **10**. From these data it becomes apparent that the ligands X and Y have a profound effect on the kinase inhibition properties in this class of compounds and the small library of organoiridium complexes **7**-**11** already spans across an activity range of almost one order of magnitudes. This observation is consistent with previous studies of organometallic protein kinase inhibitors which revealed the importance of the ligands oriented perpendicular to the pyridocarbazole moiety (ligands A and C in Figure 1) for kinase binding, especially the ligand pointing towards the glycine-rich-loop (ligand A in Figure 1) (Bregman H., 2006). Our results thus demonstrate that oxidative addition provides a convenient synthetic tool to quickly scan ligands in the positions perpendicular to the pyridocarbazole moiety, which to our opinion is a unique feature of this iridium scaffold.

For the purpose of designing compounds as molecular probes for chemical biology, the selectivity of a compound is one of its most important single features. This is especially a challenge for members of large enzyme families such as protein kinases with more than 500 putative protein kinase genes encoded in the human genome. Nevertheless, iridium complex **7** displays a remarkable degree of selectivity for Flt4 over other protein kinases. Figure 3 shows the results of a screening of complex **7** against a panel of 229 human wild type protein kinases at a concentration of 100 nM (10 µM ATP). Out of the 229 protein kinases, 224 kinases remained an activity of more than 50%, including other members of the VEGFR family such as VEGFR1 (also known as Flt1, 62% activity at 100 nM **7**, 10 µM ATP) and VEGFR2 (also known as KDR, 95% activity at 100 nM **7**, 10 µM ATP). On the other hand, only 5 kinases, including Flt4, were inhibited by more than 50% under these conditions. IC_{50} measurements with some of these kinases at the biologically more relevant concentration of 250 µM ATP confirmed the exquisite selectivity of **7** for Flt4: Pim1 (IC_{50} = 560±8 nM) and GSK3 (measure for the more potent α-isoform: IC_{50} = 338±42 nM) are inhibited with potencies which are lower compared to the inhibition of Flt4 by **7** (IC_{50} = 125±23 nM at 250 µM ATP). Thus, it can be concluded

that the high selectivity of the organometallic complex **7** renders it a promising tool for investigating biological processes related to Flt4.

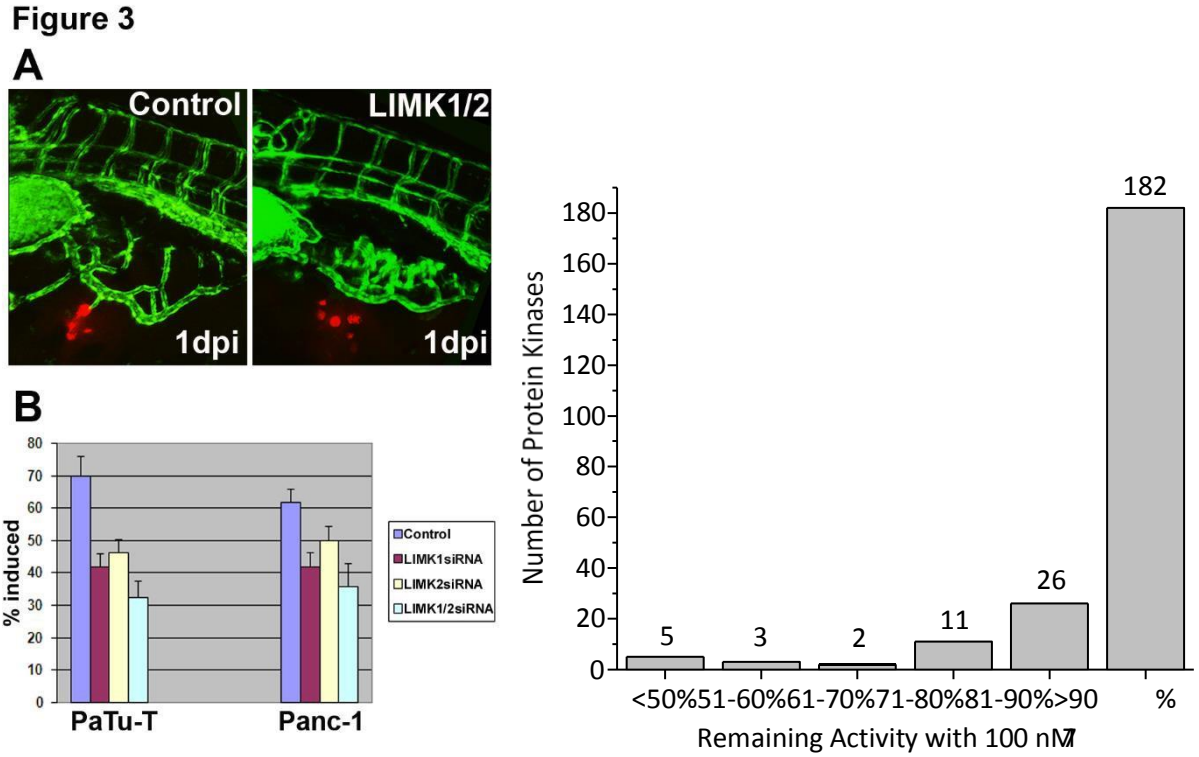


Figure 3. Selectivity profile of iridium compound **7** in a panel of 229 human wild type protein kinases. Determined by Millipore (KinaseProfiler) at a concentration of 10 µM ATP. Remaining activities are mean values from duplicate measurements. Selectivity comparison within the VEGFR family (remaining activities at 100 nM **7**): Flt1 (VEGFR1) = 62%, KDR (VEGFR2) = 95%, Flt4 (VEGFR3) = 32%. Protein kinases which were inhibited by more than 50% at 100 nM **7** are Flt4, Pim1, GSK3α, GSK3β, and MKK7β.

The receptor tyrosine kinase Flt4 plays essential roles in the development and maintenance of lymphatic vessels as well as in the development of the embryonic cardiovascular system (Karkkainen M. J. and Petrova T., 2000). In early mouse embryos it has been shown that a targeted inactivation of the Flt4 gene results in defective blood vessel formation (Dumont D.J., 1998). Similarly, it has been demonstrated that Flt4 signalling is required for vasculogenesis and angiogenesis in the developing zebrafish (Ober E. A. et al., 2004). We therefore tested the effect of complex **7** on angiogenesis and tumour-induced neovascularization in two zebrafish models (Bergamo A. et al., 2002; Ott I. et al., 2009; I. Ott et al, 2009). Accordingly, transgenic zebrafish (*Danio rerio*) embryos in which the vascular

system exhibits green fluorescence due to the expression of the green fluorescent protein (GFP) under an early endothelial promoter, were exposed to the organometallic compound **7**. The subsequent analysis with confocal fluorescence microscopy demonstrated that compound **7** induces a significant damage to the vessel formation with 79% and 100% of the zebrafish embryos being affected 3 days post fertilization at concentrations of 1 µM and 5 µM, respectively (Table 1). Figure 4 gives an example of vessel defects in zebrafish embryos 3 days post-fertilization induced by treatment with 5 µM of **7**.

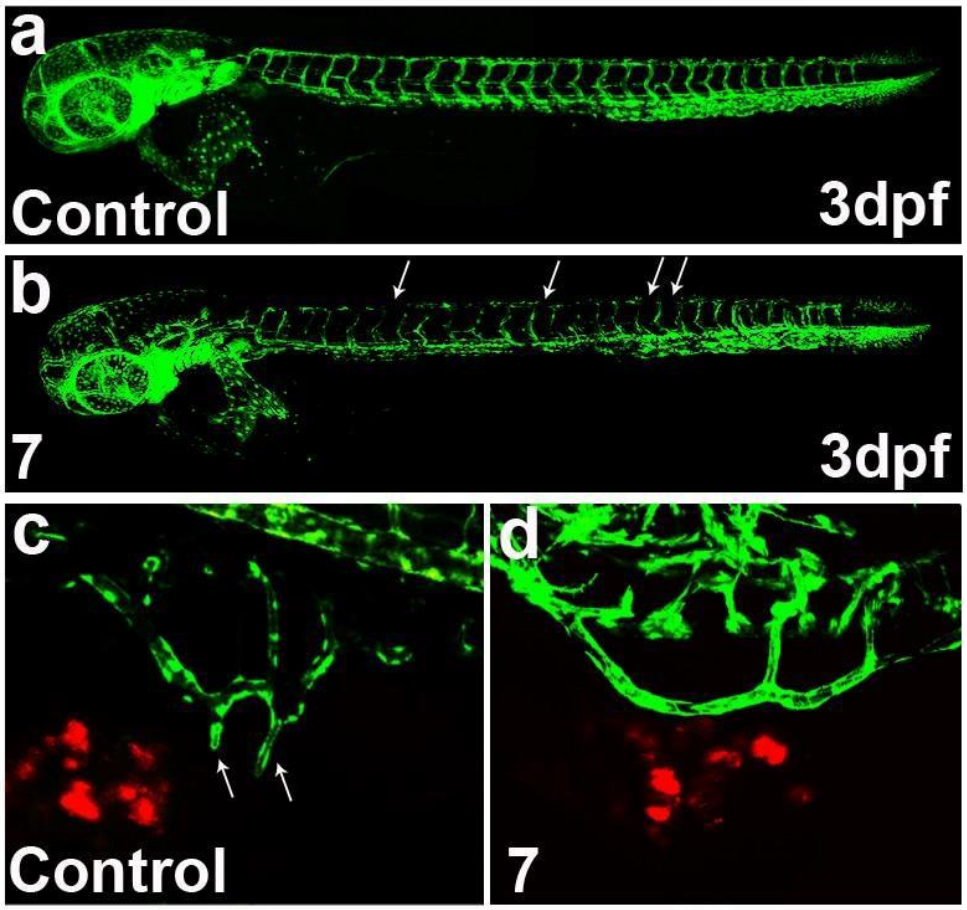


Figure 4. Effect of iridium compound **7** on angiogenesis and tumourcell-induced neovascularization in transgenic zebrafish embryos exhibiting a green fluorescent vascular system. a, b) Angiogenesis assay: Examples of laser scanning confocal microscopy images shown at 3 days post fertilization of zebrafish embryos treated with DMSO (a) or compound **7** at 5 µM (b). Vessel defects are marked with white arrows. c,d) Tumour xenotransplantation angiogenesis assay: Embryos were transplanted with human pancreatic cancer cells (red fluorescent due to CM-DiI staining) and treated with DMSO control (c) or compound **7** at 5 µM and images taken by dual laser scanning confocal microscopy at 24 hours post transplantation. The outgrowth of the subintestinal vein (SIV) in the control experiment is marked with two white arrows and suppressed in presence of compound **7**.

Table 1

Inhibition of angiogenesis and tumour-cell-induced neovascularization in developing zebrafish embryos.[a]

Compounds	Inhibition of angiogenesis (% defects / % survival)[b]	Tumour-induced neovascularization (% positive)[c]
DMSO (di-methyl sulfoxide)	0/91±2	78±4
12 (5 µM)	0/86±5	75±3
7 (1 µM)	79±5/90±2	36±1
7 (5 µM)	100±0/91±3	28±3

[a] See Supporting information for experimental details. [b] Given are the percentages of embryos with defects in dorsal longitudinal anastomic vessels (DLAV), intersegmental vessels (IV) or/and subintestinal veins (SIV) formation after 72 hours post fertilization. Percentages of surviving embryos under the experimental conditions are also indicated. Three independent experiments were peformed with 100 embryos each. No phenotypic differences were observed for the solvent control and the Nmethylated compound compared to untreated embryos. [c] Given are the percentages of embryos with induced vessel formation at 24 hours post injection of tumour cells. Two independent experiments were performed and 80 embryos were investigated for each concentration and compound. Non-transplanted zebrafish embryos do not show a similar formation of microvasculature from the subintestinal veins (data not shown).

Discussion and conclusion

New blood vessel development is an important process in tumour progression (Kerbel R. S., 2008).

Next, in order to evaluate the inhibitory effect of compound **7** on tumour-cell-induced angiogenesis, we used an *in vivo* tumour xenograft angiogenesis assay in zebrafish embryos (Nicoli, S. and Presta, M., 2007; Nicoli S. et al., 2007). This neovascularization assay allows to follow the induction of blood vessel formation by xeno-transplanted pro-angiogenic human cancer cells, in this study transplanted human pancreatic tumour cells (PaTu 8998T cells), in real-time in living zebrafish and enables the quantification of neovascularization *in vivo* and in high resolution. Revealingly, our results show inhibitory effects of **7** on tumour-cell-induced angiogenesis. For example, compound **7**, when added to the water containing the zebrafish embryos at two nonlethal concentrations, strongly inhibited the induction of new microvasculature in the zebrafish neovascularization assay (Table 1). Figure 4 visualizes the effect of compound **7**: Whereas zebrafish embryos treated with DMSO show the typical tumour-cellinduced outgrowth of the subintestinal vein (marked with two arrows in Figure 4c), the formation of new blood vessels is suppressed successfully in the presence of 5 µM of iridium compound **7** (Figure 4d).

Taken together, these data show that compound **7** has antiangiogenic properties *in vivo* and inhibits both, angiogenesis in developing zebrafish embryos as well as tumour cell induced angiogenesis. Importantly, the related organometallic complex **12** (Scheme 1), having an identical coordination sphere around the iridium(III) center but lacking the ability to inhibit protein kinases effectively due to a methylated maleimide moiety (Flt4: IC_{50} = 1520 nM at 100 µM ATP), does not show any effects at similar concentrations in these two zebrafish assays (Table 1). This pronounced difference in bioactivities between the organometallics **7** and **12** strongly suggests that it is the inhibition of protein kinases, most likely Flt4, which is responsible for the *in vivo* bioactivity of the organometallic compound **7**.

Organometallic compounds are traditionally disregarded as molecular scaffolds for the design of drug-like molecules or molecular probes due to the fear of toxicity resulting from an incompatibility of metal-carbon bonds with the biological environment. However, as shown in Table 1, the organoiridium Flt4 inhibitor **7**, containing multiple Ir-C bonds, does not affect the survival of the zebrafish embryos. Furthermore, cellular proliferation experiments with Hela cells confirm the low cytotoxicity of **7** with Hela cells being not affected significantly in the presence of 1 µM **7** over 24 hours (see Supplementary Information).

In conclusion, we herein report the first example of a protein kinase inhibitor based on an octahedral iridium complex scaffold, synthetically accessed in a stereo-selective fashion through oxidative

addition. The high *in vitro* selectivity, presumable due to the overall scaffold rigidity, combined with a lacking cytotoxicity and the distinct *in vivo* biological activity in two zebrafish model systems indicates that organoiridium complexes such as **7**, containing an octahedral metal center and multiple metal-carbon bonds, are falsley neglected as scaffolds for the development of enzyme inhibitors.

Acknowledgements

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References

Bregman H, Carroll PJ, Meggers E: Rapid access to unexplored chemical space by ligand scanning around a ruthenium center: discovery of potent and selective protein kinase inhibitors. J Am Chem Soc. 2006, 128(3):877-84.

Burgess J: Inorganic Reaction Mechanisms. 1972, volume 2. ISBN: 978-0-85186-265-1

Coe BJ, Glenwright SJ: Trans-effects in octahedral transition metal complexes. Coordination Chemistry Reviews 2000, 203, 5-80.

Dobroschke M, Geldmacher Y, Ott I, Harlos M, Kater L, Wagner L, Gust R, Sheldrick WS, Prokop A: Cytotoxic rhodium(III) and iridium(III) polypyridyl complexes: structure-activity relationships, antileukemic activity, and apoptosis induction. ChemMedChem. 2009, 4(2):177-87.

Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M, Alitalo K: Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science. 1998, 282(5390):946-9.

Karkkainen MJ, Petrova TV: Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene. 2000, 19(49):5598-605

Kerbel RS: Tumour angiogenesis. N Engl J Med. 2008, 358(19):2039-49.

Kwon TH, Kwon J, Hong JI: Signal amplification via intramolecular energy transfer using tripodal neutral iridium(III) complexes upon binding to avidin. J Am Chem Soc. 2008, 130(12):3726-7.

Maksimoska J, Feng L, Harms K, Yi C, Kissil J, Marmorstein R, Meggers E: Targeting large kinase active site with rigid, bulky octahedral ruthenium complexes. J Am Chem Soc. 2008, 130(47):15764-5.

Meggers E: Exploring biologically relevant chemical space with metal complexes. Curr Opin Chem Biol. 2007, 11(3):287-92.

Meggers E, Atilla-Gokcumen GE, Bregman H, Maksimoska J, Mulcahy S, Pagano N, Williams DS: Exploring chemical space with organometallics: ruthenium complexes as protein kinase inhibitors. Synlett 2007, 8:1177-1189

Mestroni G: Complexes of iridium(I) with 2,2'-bipyridine and 1,10-phenanthroline: Synthesis and reactions of cationic complexes with diolefins. Journal of Organometallic Chemistry. 1974, 73(1):119-127.

Mondal JU, Blake DM: Thermochemistry of the oxidative addition reactionOriginal Research Article. Coordination Chemistry Reviews 1982, 47(3):206-238

Nicoli S, Presta M: The zebrafish/tumour xenograft angiogenesis assay. Nat Protocols 2007, 2(11):2918-2923.

Nicoli S, Ribatti D, Cotelli F, Presta M: Mammalian Tumour Xenografts Induce Neovascularization in Zebrafish Embryos. Cancer Res 2007, 67(7):2927-2931.

Ober EA, Olofsson B, Mäkinen T, Jin SW, Shoji W, Koh GY, Alitalo K, Stainier DY: Vegfc is required for vascular development and endoderm morphogenesis in zebrafish. EMBO Rep. 2004, 5(1):78-84.

Ott I, Kircher B, Bagowski CP, Vlecken DH, Ott EB, Will J, Bensdorf K, Sheldrick WS, Gust R: Modulation of the biological properties of aspirin by formation of a bioorganometallic derivative. Angew Chem Int Ed Engl. 2009, 48(6):1160-3.

Scharwitz MA, Ott I, Gust R, Kromm A, Sheldrick WS: Synthesis, cellular uptake and structure-activity relationships for potent cytotoxic trichloridoiridium(III) polypyridyl complexes. J Inorg Biochem. 2008, 102(8):1623-30.

Umemoto T , Ishihara S: Power-variable electrophilic trifluoromethylating agents. S-, Se-, and Te-(trifluoromethyl)dibenzothio-, -seleno-, and -tellurophenium salt system. J. Am. Chem. Soc., 1993, 115(6):2156–2164

Vacca A, Bruno M, Boccarelli A, Coluccia M, Ribatti D, Bergamo A, Garbisa S, Sartor L, Sava G: Inhibition of endothelial cell functions and of angiogenesis by the metastasis inhibitor NAMI-A. Br J Cancer. 2002, 86(6):993-8.

Yu M, Zhao Q, Shi L, Li F, Zhou Z, Yang H, Yi T, Huang C: Cationic iridium(III) complexes for phosphorescence staining in the cytoplasm of living cells. Chem Commun (Camb). 2008, (18):2115-7.