Full Paper

Relationship Between Anticancer Activity and Stereochemistry of Saldach Ligands and their Iron(III) Complexes

Dedicated to Professor H. Schönenberger on his 85. birthday

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(*R*,*R*)-, (*S*,*S*)- and (*R*,*S*)-*N*,*N*′-bis(salicylidene)-1,2-diaminocyclohexane (saldach) and their iron(III) complexes were screened for anticancer activity against MCF-7 and MDA-MB 231 breast cancer as well as HT-29 colon carcinoma cells. Antiproliferative effects depended on the presence of the central atom iron but were independent on the configuration at the saldach ligand. While the free ligands were inactive, the iron(III) derivatives displayed anticancer activity within a concentration range of 1 to 5 μM irrespective of the used cell line. At 5 μM they were even more active than *cis*-platin. A mode of action comparable to *cis*-platin can be excluded because it is very likely that the DNA is not the primary target of [Fe^{III}(saldach)] complexes.

Keywords: Anticancer activity / HT-29 / Iron saldach complexes / MCF-7 / MDA-MB 231

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Introduction

Transition metal compounds containing an 1,2-diaminocyclohexane (DACH) building block have been used as catalysts for many years [1, 2]. The use in pharmaceutical research increased with the observation of Kidani *et al*. in the early 1980s that platinum complexes bearing DACH as carrier ligand exhibited excellent antitumor activity [3, 4]. Already at the beginning of the studies, a clear dependence of the tumor-growth inhibiting effects on the configuration of the DACH ligand was observed: The meso form was less active than the racemic form and the (*R*,*R*)-enantiomer was more active than its (*S*,*S*)-congener, *e.g*. against the L1210 leukemia of the mouse. Optimization of the leaving group led to [(*R*,*R*)-1,2-diamino-cyclohexane]-oxalato-platinum(II) which is now established as oxaliplatin in the systemic treatment of advanced colorectal carcinoma both, as a single agent and in combination regimens. Although the DACH ligand represents a promising carrier ligand, there is only few information about non-platinum 1,2-diaminocyclohexane complexes displaying anticancer activity [5–7]. Therefore, we modified DACH as well as its phenylenediamine congener in such a way that they were suitable to obtain metal complexes.

We already demonstrated that the [*N*,*N*′-bis(salicylidene)-1,2-phenylenediamine]iron(II/III) complexes strongly reduced cell proliferation more effectively than *cis*-platin. This was ascribable to the presence of iron as the central atom in combination with an intact phenylenediamine moiety. The reduction of the ligand resulting in rac-*N*,*N*′-bis(salicylidene)-1,2-cyclohexanediamine strongly reduced the efficacy of the iron complexes.

The contribution of the DACH ligands chirality to the anticancer activity of oxaliplatin prompted us to study its influence on the cytotoxic properties of [Fe^{III}(saldach)Cl] complexes (see Fig. 1) [8]. We selected the oxidation state +III because an earlier study demonstrated nearly identical effects of iron(II) and iron(III) complexes. Furthermore, we also investigated the cytotoxicity of the free ligands, because Gao *et al*. recently identified related chiral *N*,*N*′-bis-salicyl-1,2-diaminocyclohexane compounds as effective anticancer agents in the down regulation of Bcl-2-family gene expression in human breast cancer cells [9].

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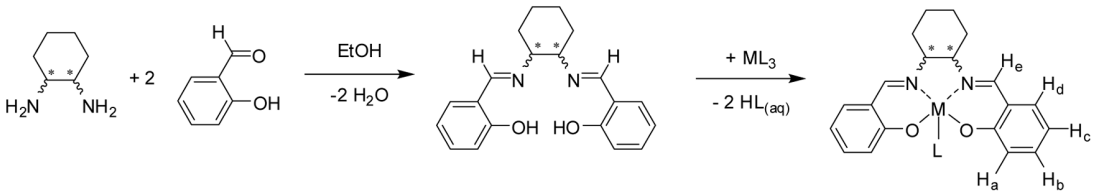
Abbreviations: 1,2-diaminocyclohexane (DACH); poly{ADP-ribose} polymerase (PARP); reactive oxygen species (ROS)



1,2-diaminocyclohexane	M^{III}	L^-	compound
rac-*trans*-C_6H_{10}	-	-	**1**
(*R*,*R*)-*trans*-C_6H_{10}	-	-	**2**
(*S*,*S*)-*trans*-C_6H_{10}	-	-	**3**
(*R*,*S*)-*cis*-C_6H_{10}	-	-	**4**
rac-*trans*-C_6H_{10}	Fe	Cl	**5**
(*R*,*R*)-*trans*-C_6H_{10}	Fe	Cl	**6**
(*S*,*S*)-*trans*-C_6H_{10}	Fe	Cl	**7**
(*R*,*S*)-*cis*-C_6H_{10}	Fe	Cl	**8**

Figure 1. Synthesized saldach ligands **1**-**4** and their [Fe^{III}(saldach)Cl] complexes **5**-**8**.

Results and discussion

Synthesis and structural characterization

Saldach ligands as well as the iron complexes (see Fig. 1) were synthesized as described previously [8, 10].

The formation of the Schiff bases was confirmed by the presence of a ν(C=N) at about 1630 cm^{-1} in the IR spectra (see Experimental). Furthermore, the imine nitrogen is part of an H-bond from the phenolic hydrogen (O-H···N=C; salicylic effect) resulting in a broadening of the OH stretching vibration in the region between 3500 to 2500 cm^{-1} [11]. In the ^{1}H-NMR spectra of **1** to **4**, recorded in DMSO-d_6 and $CDCl_3$, the imine proton appeared at δ = 8.49 (**1**), 8.26 (**2**, **3**), and 8.29 (**4**), while the OH resonance is located at δ = 13.33 (**1**), 13.32 (**2**, **3**), and 13.41 (**4**), respectively.

After coordination, NMR analysis is impossible due to the paramagnetism of the iron complexes. In the IR spectra, the stretching vibration of the imine bond is shifted by Δν = 18 cm^{-1} to lower frequencies. This shift is in accordance with that observed for [*N*,*N*′-bis(salicylidene)-1,2-phenylenediamine]iron(II/III) and confirmed the coordination of the imine via the nitrogen to iron. A further chelate ring is built due to the binding of the phenolic oxygen to iron indicated by the disappearance of the broad O-H···N=C band in the IR spectra.

Biological activity

Biological properties were evaluated in relation to *cis*-platin, which showed cytostatic activity against MCF-7 and MDA-MB 231 cells (T/C_{corr} = 3%, respectively). On HT-29 cells it was less active (T/C_{corr} = 35%) (see Fig. 2).

Free ligands as well as their complexes were administered at equimolar concentrations. At 0.5, 1, and 5 μM all ligands were completely inactive (data not shown), while the complexes showed concentration-dependent antiproliferative effects. Compared to *cis*-platin, the cytotoxicity was somewhat higher. The time response curve of *cis*-platin is characterized by a maximum effect at the end of the test (after an incubation time of 150 to 220 h), whereas the highest antiproliferative effects of the iron complexes were detected already after 48 to 72 h. Therefore, it is necessary to evaluate the cytotoxicity in time-respond curves instead of concentration-respond curves as realized for the calculation of IC_{50} values.

The effects depended on the used cell line. Cytostatic or low cytocidal effects were caused at the highest concentration of 5 μM. Significantly reduced cell growth was

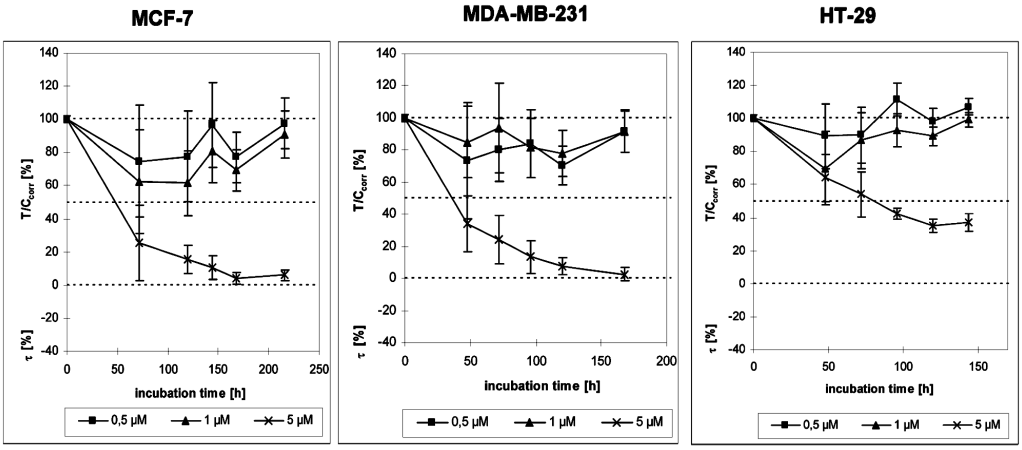


Figure 2. Antiproliferative effects of *cis*-platin on the human MCF-7 and MDA-MB 231 breast cancer as well as the human HT-29 colon carcinoma cell lines.

caused at 1 µM only in the case of (*R*,*R*)-*trans*-[Fe^{III}(saldach)Cl] complex **6** against the MDA-MB 231 cell line. These very steep concentration-activity curves indicated nonspecific cell toxic effects independent of the configuration at the DACH ligand (see Fig. 3).

Gao *et al*. investigated the influence of *N*,*N*′-bis-salicyl-1,2-diaminocyclohexane isomers on the growth of tumor cells, *e.g*. the MCF-7 breast cancer cells. The (*R*,*R*)-configurated isomer (IC_{50} = 0.4 µM) was somewhat more active than its (*S*,*S*)-enantiomer (IC_{50} = 0.6 µM) and both were remarkably more cytotoxic than the (*R*,*S*)-isomer. Furthermore, the (*R*,*R*)-enantiomer displayed in SRB (growth inhibition assay using Sulforhodamine B staining technique [12]) and colony-formation assays a significantly greater cytotoxic activity toward MCF-7 cells than non-tumorigenic MCF-10A breast cells and was an extremely efficient regulator of anti-apoptotic genes, Bcl-xL, Bcl-2, and the cell cycle related gene, cyclin D1 [7].

Exchange of the amine by an imine bond completely abolished the cytotoxic properties. The *N*,*N*′-bis(salicylidene)-1,2-cyclohexanediamines **1**–**4** did not influence the growth of MCF-7, MDA-MB 231, and HT-29 cells. This effect might be the consequence of a reduced degree of freedom which hinders the interaction with specific targets.

The imine bond and the salicyl effect caused a very rigid conformation with rotation possible only around the N-C axis at the cyclohexane. The latter is prohibited upon coordination to iron. The flexibility of the build five-membered iron ethylenediamine chelate is marginal and allows only an interconversion between a δ- and a λ-form as it is well known from platinum(II) complexes. [3, 13–15] A further dynamic process which is independent on the coordination to iron suffers from the cyclohexane ring: It changes between chair-chair or chair-boat conformations (see Fig. 4).

Configurations at the asymmetric C atoms determine the orientation of the cyclohexane ring relative to the iron plane (see Fig. 4).

In the case of (*R*,*R*)- and (*S*,*S*)-configurated compounds a coplanarity of the most likely chair conformation and the chelate ring could be assumed. A change to the (*R*,*S*)-configuration led to a nearly perpendicular orientation both, in the chair and the boat conformation.

FA very complex mode of action was already discussed for [rac-*N*,*N*′-bis(salicylidene)-1,2-cyclohexanediamine]iron(II/III). Generation of reactive oxygen species (ROS) or DNA interaction followed by induction of apoptosis indicated by PARP cleavage experiments (PARP = an 116-kDa nuclear poly{ADP-ribose} polymerase, appears to be involved in DNA repair and serves as a marker of cells undergoing apoptosis [16]) would be a possible explanation for the cytotoxicity which is depended on the presence of an iron center.

The 3D structure of the [Fe^{III}(saldach)Cl] complexes (see Fig. 4) allows a DNA intercalation by the iron salene moiety. If this is realized, a clear difference in antiproliferative potency should be observed in the *in-vitro* assay (see Fig. 3). The racemic form and the separated (*R*,*R*)- and (*S*,*S*)-enantiomers are plane structures and should be better attached to the DNA compared to the (*R*,*S*)-isomer. The cyclohexane ring, located perpendicular to the iron salene, would hinder or even prevent the intercalation between nucleobases. From Fig. 3, however, it is obvious that neither conformation nor configuration played a critical role for cell-growth inhibiting effects. Since DNA

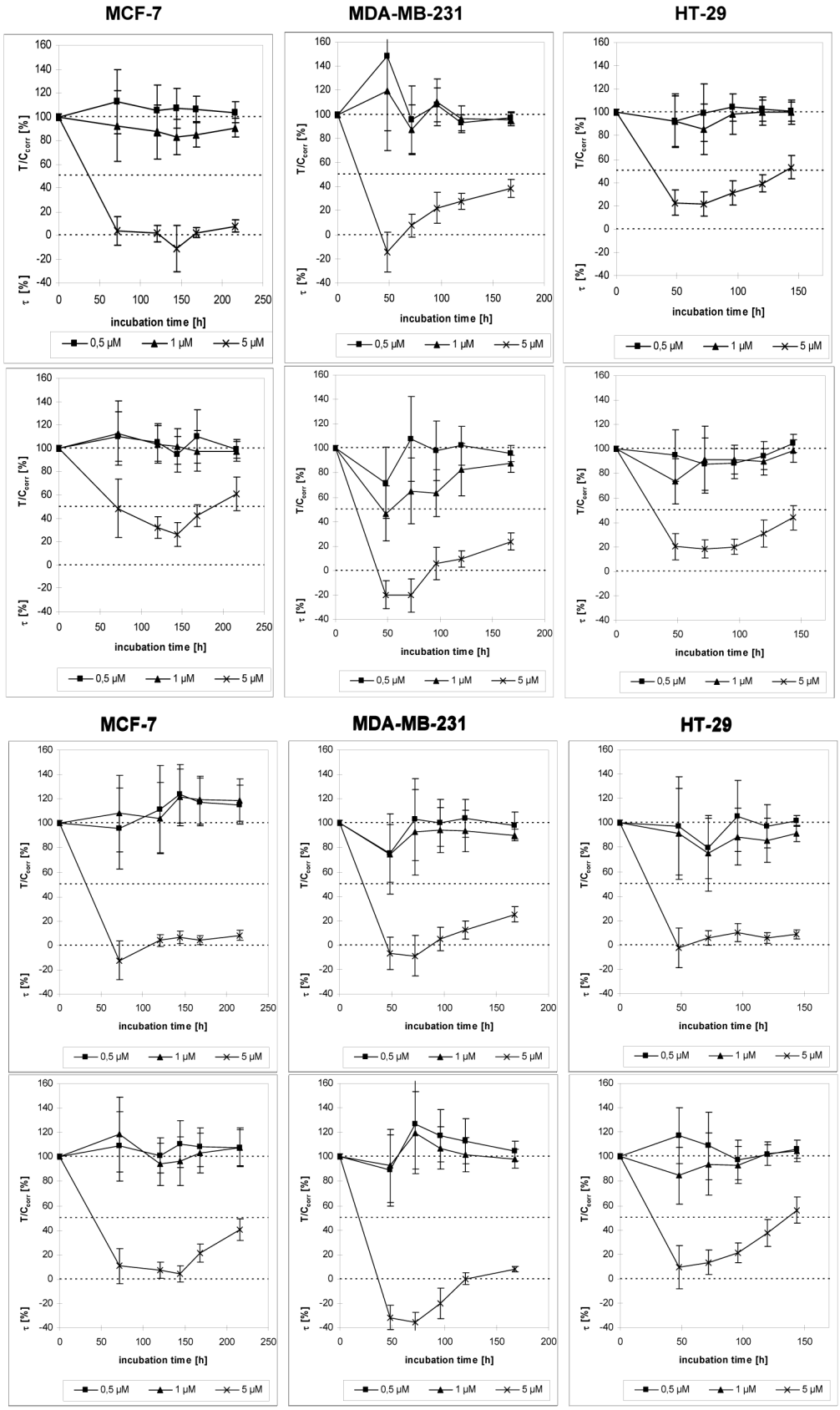


Figure 3. Time-dependent analysis of antiproliferative effects of the [Fe^{III}(saldach)Cl] complexes **5**, **6, 7** and **8** on the human MCF-7 and MDA-MB 231 breast cancer and the HT-29 colon carcinoma cell lines.

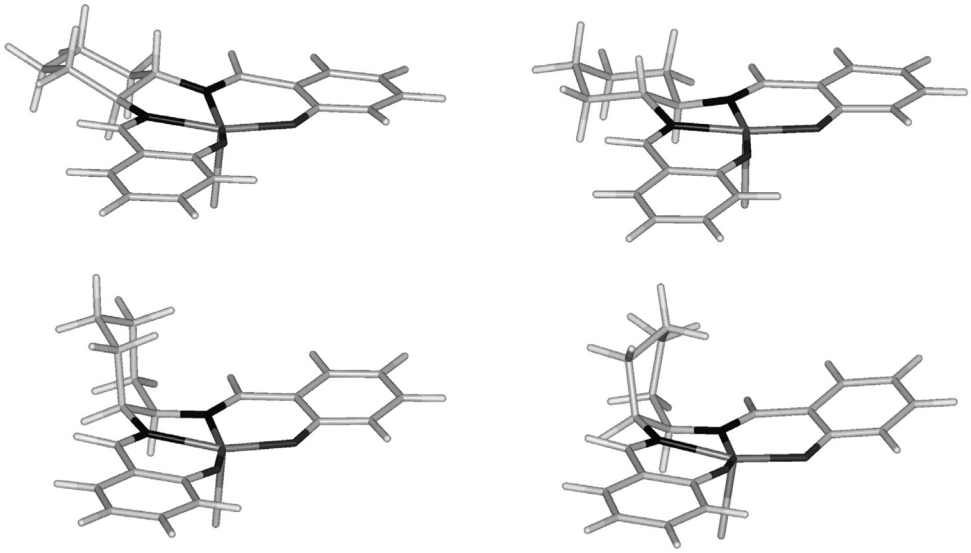


Figure 4. Conformations of the (*R*,*R*)-isomer **6** (above) and the (*R*,*S*)-isomer **8** (below); interconversion of the cyclohexane ring.

is a chiral macromolecule and differentiates between enantiomeric cytostatics, it is not the target of the complexes **5**–**8** [17, 18].

Such considerations were subject of many SAR studies on oxaliplatin and related compounds [3]. Although the DNA is the accepted target of platinum complexes, it can be excluded in the case of our iron salene complexes (see also [8]). Cell-death promoting effects were unspecific, depended on the presence of an iron centre and independent of the conformation/configuration of the saldach ligand.

It is well known that iron salene complexes are able to generate reactive oxygen species (ROS) [8], able to damage essential signal cascades in the cells. The generation of ROS in MCF-7 was already verified for the racemic complex **5**. Therefore, in a further study, we will look for the real intracellular target of iron salene complexes and try to optimize the cytotoxic potency. If cell damage is caused by redox reactions, it will be of interest to investigate how substituents at the aromatic rings coordinated to iron influence the redox behavior and the cytotoxicity. First results with methoxy groups located at the salicylidene moieties were promising [19].

Experimental

Chemistry

General procedures

The following instrumentation was used: ^{1}H-NMR, Bruker ADX 400 spectrometer (Bruker Bioscience, USA) at 400 MHz (internal standard, TMS); EI-MS spectra, CH-7A Varian (70 eV; Varian Inc., Palo Alto, CA, USA); IR spectra (KBr pellets), Perkin-Elmer model 580 A (Perkin Elmer, USA). Elemental C, H, N analyses were carried out with a Perkin-Elmer 240 B and 240 C elemental analyzer. Chemicals were obtained from Sigma Aldrich (Germany) and were used as received.

Synthesis of ligands (method A)

Schiff bases were prepared involving reaction of salicylaldehyde with the corresponding diamine (2:1 molar ratio) in ethanol. The imines were filtered, washed with ethanol, dried over P_2O_5, and used without further purifications.

Synthesis of ligands (method B)

Schiff bases were prepared according to a published procedure with minor modifications [10]. The respective 1,2-diammoniumcyclohexane mono-(+/−)-tartrate salt (4.60 mmol) and potassium carbonate (9.95 mmol) were dissolved in a mixture of distilled water (30 mL) and ethanol (10 mL). The mixture was heated with salicylaldehyde (9.95 mmol) in ethanol (10 mL) under reflux for 2 h. Subsequently, the yellow slurry was cooled to 5°C or lower in an ice bad, filtered and dissolved in CH_2Cl_2 (30 mL). The organic layer was washed twice with water (10 mL) and brine (20 mL), dried over Na_2SO_4, and the solvent was removed *in vacuo*. A yellow solid was obtained after crystallization in the refrigerator.

Synthesis of the complexes

The complexes were prepared by reaction of an ethanolic solution of $FeCl_3 \times 6\ H_2O$ with a boiling solution of the respective Schiff base in ethanol (1:1 molar ratio) under reflux for 1 h. The mixture was allowed to cool to room temperature, the precipitated complexes were filtered off, washed with ethanol, and dried over P_2O_5.

rac-trans-N,N′-Bis(salicylidene)-1,2-cyclohexanediamine ***1***

Compound **1** was obtained from *cis/trans*-1,2-cyclohexanediamine (3.00 g, 26.3 mmol) and salicylaldehyde (6.42 g, 52.5 mmol) (method A).

Yield: 4.76 g (14.76 mmol, 56%); crystalline yellow powder; m.p.: 114–115°C [20]; $[\alpha]_D^{20}$ = 0 (EtOH, c = 0.1), IR (KBr) ν [cm^{-1}]: 3433 br, 3011 w, 3068 w, 2993 m, 1630 s, 1501 m, 1280 s; ^{1}H-NMR (DMSO-d_6) δ: 13.33 (s, 2H, Ar-OH), 8.49 (s, 2H, NC*H*), 7.36–7.32 (dd, ^3J = 7.7 Hz, ^4J = 1.6 Hz, 2H, H_d), 7.29–7.25 (td, ^3J = 7.7 Hz, ^4J = 1.6 Hz, 2H, H_b), 6.85–6.80 (m, ^3J = 7.5 Hz, ^4J = 1.0 Hz, 4H, H_a + H_c), 3.42–3.36 (m, 2H), 1.90–1.79 (m, 4H), 1.63–1.61 (m, 2H), 1.48-1.43 (m, 2H); MS (EI, 75°C) *m/z*: 322 (57%) [M^+]. Anal. calcd. for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.52; H, 6.74; N, 8.53.

(R,R)-trans-N,N′-Bis(salicylidene)-1,2-cyclohexanediamine ***2***

Compound **2** was obtained from (*R*,*R*)-1,2-diammoniumcyclohexane mono-(+)-tartrate salt (1.23 g, 4.65 mmol) and salicylaldehyde (1.21 g, 9.91 mmol) (method B).

Yield: 1.15 g (3.57 mmol, 77%); crystalline yellow solid; $[\alpha]_D^{20}$ = −444.0 (EtOH, c = 0.1); IR (KBr) ν [cm^{-1}]: 3453 br, 3052 w, 2935 m, 2856 m, 1630 s, 1497 m, 1278 s; ^{1}H-NMR ($CDCl_3$) δ: 13.32 (s, 2H, Ar-OH), 8.26 (s, 2H, NC*H*), 7.26–7.22 (td, ^3J = 6.98 and 6.86 Hz, ^4J = 1.12 and 1.21 Hz, 2H, H_b), 7.16–7.13 (dd, ^3J = 7.70 and 7.60 Hz, ^4J = 1.70 and 1.62 Hz, 2H, H_d), 6.89–6.87 (d, ^3J = 8.27 Hz, 2H, H_a), 6.81–6.77 (td, ^3J = 6.63 and 7.52 Hz, ^4J = 1.13 and 0.92 Hz, 2H, H_c), 3.35–3.28 (m, 2H), 1.96–1.88 (m, 4H), 1.77–1.69 (m, 2H), 1.50–1.45 (m, 2H); MS (EI, 90°C) *m/z*: 322 (60%) [M^+]. Anal. calcd. for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.69; H, 7.07; N, 8.78.

(S,S)-trans-N,N′-Bis(salicylidene)-1,2-cyclohexanediamine ***3***

Compound **3** was obtained from (*S*,*S*)-1,2-diaminocyclohexane (0.22 g, 1.93 mmol) and salicylaldehyde (0.47 g, 3.86 mmol) (method A).

Yield: 0.24 g (0.75 mmol, 39%); yellow solid; $[\alpha]_D^{20}$ = + 377.4 (EtOH, c = 0.1); IR (KBr) ν [cm^{-1}]: 3443 br, 3052 w, 2935 m, 2857 m, 1631 s, 1497 m, 1278 s. ^{1}H-NMR ($CDCl_3$) δ: 13.32 (s, 2H, Ar-OH), 8.26 (s, 2H, NC*H*), 7.26–7.21 (td, ^3J = 7.16 and 7.73 Hz, ^4J = 1.47 Hz, 2H, H_b), 7.16–7.14 (dd, ^3J = 7.64 Hz, ^4J = 1.24 Hz, 2H, H_d), 6.90–6.87 (d, ^3J = 8.23 H, 2H, H_a), 6.81–6.77 (td, ^3J = 7.49 and 7.42 Hz, 2H, H_c), 3.38–3.26 (m, 2H), 1.96–1.82 (m, 4H), 1.77–1.72 (m, 2H), 1.54–1.43 (m, 2H); MS (EI, 90°C) *m/z*: 322 (45%) [M^+]. Anal. calcd. for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.69; H, 6.65; N, 8.45.

(R,S)-cis-N,N′-Bis(salicylidene)-1,2-cyclohexanediamine ***4***

Compound **4** was obtained from (*R*,*S*)-1,2-diaminocyclohexane (0.96 g, 8.39 mmol) and salicylaldehyde (2.05 g, 16.80 mmol) (method A).

Yield: 2.41 g (7.47 mmol, 89%); crystalline yellow solid; IR (KBr) ν [cm^{-1}]: 3437 br, 3049 w, 2933 s, 2858 m, 1629 s, 1500 m, 1281 s; ^{1}H-NMR ($CDCl_3$) δ: 13.41 (s, 2H, Ar-OH), 8.29 (s, 2H, NC*H*), 7.23–7.21 (dd, ^3J = 8.65 and 7.85 Hz, ^4J = 0.83 and 1.63 Hz, 2H, H_d), 7.19–7.16 (td, ^3J = 7.83 Hz, ^4J = 1.68 and 1.42 Hz, 2H, H_b), 6.86–6.84 (d, ^3J = 8.37 Hz, 2H, H_a), 6.80–6.77 (td, ^3J = 7.06 and 7.22 Hz, ^4J = 0.92 Hz, 2H, H_c), 3.56–3.54 (m, 2H), 1.95–1.80 (m, 4H), 1.73–1.68 (m, 2H), 1.56–1.44 (m, 2H); MS (EI, 100°C) *m/z*: 322 (48%) [M^+]. Anal. calcd. for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.39; H, 6.85; N, 8.45.

Iron(III) saldach complex ***5***

Compound **5** was obtained from *rac-trans-N,N′*-bis(salicylidene)-1,2-cyclohexanediamine (100.00 mg, 0.31 mmol) and iron(III) chloride hexahydrate (84.70 mg, 0.31 mmol).

Yield: 90.00 mg (0.22 mmol, 70%); black solid; IR (KBr) ν [cm^{-1}]: 2924 m, 1612 s, 1543 m, 1312 m; MS (EI, 275°C) *m/z*: 411 (64%) [M^+], 376 (100%) [$M^{+\bullet}$−Cl], 56 (20) [Fe]. Anal. calcd. for $C_{20}H_{20}N_2O_2$FeCl: C, 58.35; H, 4.90; N, 6.80. Found: C, 58.52; H, 4.80; N, 6.82.

Iron(III) saldach complex ***6***

Compound **6** was obtained from (*R*,*R*)-*N,N′*-bis(salicylidene)-1,2-cyclohexanediamine (103.00 mg, 0.32 mmol) and iron(III) chloride hexahydrate (86.00 mg, 0.32 mmol).

Yield: 101.00 mg (0.24 mmol, 77%); black solid; IR (KBr) ν [cm^{-1}]: 2925 s, 1614 s, 1543 m, 1312 m; MS (EI, 250°C) *m/z*: 411 (64%) [M^+], 376 (100%) [$M^{+\bullet}$−Cl], 56 (20) [Fe]. Anal. calcd. for $C_{20}H_{20}N_2O_2$FeCl: C, 58.35; H, 4.90; N, 6.80. Found: C, 58.36; H, 5.19; N, 6.99.

Iron(III) saldach complex ***7***

Compound **7** was obtained from (*S*,*S*)-*N,N′*-bis(salicylidene)-1,2-cyclohexanediamine (157.00 mg, 0.49 mmol) and iron(III) chloride hexahydrate (130.80 mg, 0.49 mmol).

Yield: 79.00 mg (0.22 mmol, 70%); black solid; IR (KBr) ν [cm^{-1}]: 2936 m, 1612 s, 1544 m, 1312 m; MS (EI, 275°C) *m/z*: 411 (64%) [M^+], 376 (100) [$M^{+\bullet}$−Cl], 56 (20) [Fe]. Anal. calcd. for $C_{20}H_{20}N_2O_2$FeCl: C, 58.35; H, 4.90; N, 6.80. Found: C, 58.76; H, 5.38; N, 6.98.

Iron(III) saldach complex ***8***

Compound **8** was obtained from (*R*,*S*)-*N,N′*-bis(salicylidene)-1,2-cyclohexanediamine (178.00 mg, 0.55 mmol) and iron(III) chloride hexahydrate (148.70 mg, 0.55 mmol).

Yield: 123.00 mg (0.30 mmol, 54%); black solid; IR (KBr) ν [cm^{-1}]: 2928 w, 1612 s, 1543 m, 1301 m; MS (EI, 275°C) *m/z*: 411 (64) [M^+], 376 (100) [$M^{+\bullet}$−Cl], 56 (20) [Fe]. Anal. calcd. for $C_{20}H_{20}N_2O_2$FeCl: C, 58.35; H, 4.90; N, 6.80. Found: C, 58.52; H, 4.80; N, 6.82.

Biochemicals, chemicals, and materials

Cell culture conditions

The human MCF-7 and MDA-MB 231 breast cancer and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection (ATCC). Both cell lines were maintained as a monolayer culture in L-glutamine containing Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose (PAA Laboratories GmbH, Austria), supplemented with 10% fetal calf serum (FCS; Gibco, Germany) using 25 cm^2 culture flasks in a humidified atmosphere (5% CO_2) at 37°C. The cell lines were passaged twice a week after previous treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (0.02% EDTA; Boehringer, Germany).

In-vitro chemosensitivity assay

The *in-vitro* testing of the substances for antitumor activity in adherent growing cell lines was carried out on exponentially dividing human cancer cells according to a previously published microtiter assay [21, 22]. Exponential cell growth was ensured

during the whole time of incubation. Briefly, 100 µL of a cell suspension was placed in each well of a 96-well microtiter plate at 7200 cells/mL (MCF-7), at 3000 cells/mL (MDA-MB 231), and at 1600 cells/mL (HT-29) of culture medium and incubated at 37°C in a humidified atmosphere (5% CO_2) for 3 d (MCF-7) and for 2 d (MDA-MB 231 and HT-29), respectively. By removing the medium and adding 200 µL of fresh medium containing an adequate volume of a stock solution of the metal complex, the desired test concentration was obtained. *Cis*-platin was dissolved in DMF while DMSO was used for all other compounds. Eight wells were used for each test concentration and for the control, which contained the corresponding amount of DMF and DMSO, respectively. The medium was removed after reaching the appropriate incubation time. Subsequently, the cells were fixed with a solution of 1% (v/v) glutaric dialdehyde in phosphate buffered saline (PBS) and stored under PBS at 4°C. Cell biomass was determined by means of a crystal violet staining technique as described earlier [21, 22]. The effectiveness of the complexes is expressed as corrected T/C_{corr} [%] or t [%] values according to the following equations:

$$\text{cytostatic effect: } T/C_{corr}\,[\%] = [(T - C_0)/(C - C_0)] \times 100 \quad (1)$$

$$\text{cytocidal effect: } t\,[\%] = [(T - C_0)/C_0] \times 100 \quad (2)$$

whereby T (*test*) and C (*control*) are the optical densities at 590 nm of crystal violet extract of the cells in the wells (*i.e.* the chromatin-bound crystal violet extracted with ethanol (70%)) with C_0 being the density of the cell extract directly before treatment. For the automatic estimation of the optical density of the crystal violet extract in the wells, a microplate autoreader (Flashscan S 12; Analytik Jena, Germany) was used.

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The authors have declared no conflict of interest.

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