Auranofin, Et_3PAuCl and Et_3PAuI exert high in vitro cytotoxic effects toward colorectal cancer cell lines: a comparative chemical, biological and mechanistic study.

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ABSTRACT: The solution behavior of Et_3PAuI, Et_3PAuCl and AF as well as their interactions with a hen egg white lysozyme and a standard single strand oligonucleotide were comparatively analyzed through NMR spectroscopy and ESI-MS. Binding ability of the three complexes toward ds-DNA was also assessed by ethidium bromide displacement and viscometric tests. The cytotoxic effects toward two representative colorectal cancer cell lines were found to be strong and similar in the three cases and a good correlation could be established between the cytotoxicity and the ability to inhibit thioredoxin reductase. Overall, a very similar profile emerges for Et_3PAuI and Et_3PAuCl, that retain the potent cytotoxic effects of Auranofin, while showing some peculiar features. These results demonstrate that the presence of the thiosugar moiety is not mandatory for the pharmacological action, suggesting that the tuning of some relevant chemical properties such as lipophilicity could be exploited to improve bioavailability, with no loss of the pharmacological effects.

Auranofin [2,3,4,6-tetra-o-acetyl-L-thio-β-D-glyco-pyranosato-S-(triethyl- phosphine)-gold(I)] (AF) is a clinically established oral chrysotherapeutic agent that is used for the treatment of some severe forms of rheumatoid arthritis.[1] During the last few years, this drug has attracted renewed attention in the medicinal chemistry scientific community as a prospective anticancer and antimicrobial agent according to innovative drug repurposing strategies.[2-4] In particular, AF is currently undergoing two distinct clinical trials in the US as an anticancer agent.[5,6] We thought that selective and limited chemical modifications of AF might lead to a modulation and hopefully an improvement of its pharmacological profile. To this regard it is worth reminding that Frank Shaw, on the ground of similar arguments, prepared and characterized a few years ago selenoauranofin, a derivative of AF where the thiosugar ligand is replaced by the corresponding selenosugar ligand and obtained remarkable biological results.[7] Accordingly, we decided to prepare a derivative of AF where the thiosugar ligand is replaced by one iodide ligand and to test this compound in comparison to AF and its commercially available chloride analogue. In principle, replacement of the thiosugar or chloride ligand with iodide should afford a compound of increased lipophilicity, thus enhancing drug bioavailability; at the same time, substitution of such a large ligand as thioglucose tetracetate with a monoatomic ligand, *i.e.* iodide, should not affect substantially the drug's pharmacological profile as the thiosugar ligand is believed to act mainly as a carrier ligand and, also, as a good leaving group, while the Et_3PAu^+ moiety is assumed to be the "true pharmacophore". In nice agreement with this view, previous studies showed that Et_3PAuCl manifests biological properties similar though not identical to those of AF.[8]

Et_3PAuI was synthesized starting from commercially available Et_3PAuCl. First, Et_3PAuCl was treated with an excess of KI in ethanol at RT. After 3 h, the mixture was dried and the resulting white solid kept at -20° C overnight. The product was then extracted with dichloromethane and the recombined organic phase was washed with water and dried over $MgSO_4$. After precipitation in pentane, white crystals of Et_3PAuI were collected and dried.

Et_3PAuI was characterized by elemental analysis and NMR confirmed the purity of the synthesized compound (see S2 ESI). A log*P* value of 4.6 was found for Et_3PAuI making this

complex by far the most lipophilic of the series. Indeed, log*P* values of 1.7 and 1.6 were respectively measured for Et_3PAuCl and AF (S14 in supporting material for details on the method for log*P* determination).

Single crystals of Et_3PAuI, suitable for X-ray diffraction studies, were obtained by adding pentane to a concentrated solution of the compound in dichloromethane. The solution was kept at -20 °C for one week. After this time needle-shaped crystals were formed (Figure 1, S16 in ESI for crystallographic data).

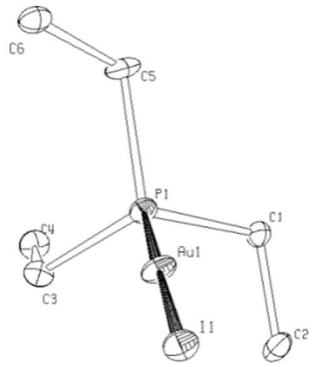

Figure 1. X ray structure of complex Et_3PAuI. The gold(I) ion is linearly coordinated to the I(1) and P(1) atoms, being the distances 2.5898(1) and 2.268(2) Å respectively. The angle P(1)-Au(1)-I(1) is 178.89(6)°.

The crystal structure was solved to 0.73 Å resolution. The geometry as well as the distances reported in Fig. 1 are in good agreement with bond lengths and angles found in similar compounds retrieved from the CSD (v. 5.37 February 2016). It is worth reminding, that the Au-I bond length generally increases as the hindrance of the substituents of the P atom decreases (P-*t*-Bu_3, P-i-Pr_3, P-Et_3, 2.56, 2.58 and 2.59 Å respectively).[9]

Afterward, Et_3PAuI was investigated for its chemical and biological properties in solution in comparison to AF and Et_3PAuCl. The solution behavior of the three species was mainly assessed by ^{31}P NMR spectroscopy. The three compounds were solubilized in trizma Base/CH_3COOH, 6 mM, in the presence of 250 µL H_2O; 650 µL CH_3OH; 200 µL CD_3OD, pH 7 and analyzed at increasing time intervals. Notably, all three compounds manifest a high stability for several hours with no evidence of ligand detachment. Indeed, changes in the ^{31}P NMR spectra could only be detected after very long incubation times. For Et_3PAuI, no changes were seen after 72 h; however, after one week of incubation, beside the signal falling at 41 ppm (assigned to the phosphorous in the neutral complex), a new signal of low intensity appeared at 47 ppm, in the spectrum of Et_3PAuI. This new signal is tentatively attributed to phosphorous in the cationic mono-charged complex $Au(PEt_3)_2^+$, that is likely formed through rearrangement, according to the equilibrium:[10,11]

$$2(Et)_3PAuI \rightleftharpoons Au(PEt_3)_2^+ + AuI_2^-$$

Scheme 1. Ligand scrambling reaction of Et_3PAuI.

The signal increases in intensity until day 14; no further changes in intensity were detected afterward, even after 35 days, indicating that the reaction has reached its equilibrium (Fig. 2, S4-S8 in supporting material for details and spectra).

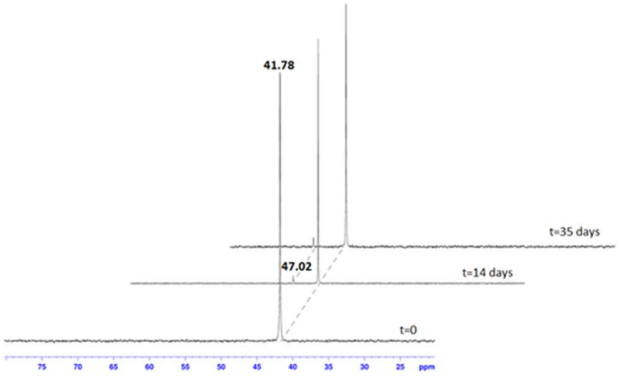

Figure 2. ^{31}P NMR spectra (Trizma Base/CH_3COOH 6 mM in a mixture of 250 µL H_2O; 650 µL CH_3OH; 200 µL CD_3OD, pH 7) of Et_3PAuI recorded at different time intervals.

Formation of $Au(PEt_3)_2^+$ was independently confirmed upon treating Et_3PAuI with $AgNO_3$, under the same solution conditions, and then analyzing the reaction products by ^{31}P NMR and HR ESIMS at different times intervals (S9-S10 supporting information). When recording a ^{31}P NMR spectrum immediately after treatment with $AgNO_3$ we found only a signal falling at 30.43 ppm assignable to $Et_3PAu(H_2O)^+$ species.[12] After five days, a new ^{31}P NMR spectrum was recorded on the same sample: beside the signal at 30.43 ppm, a new one at 47.85 ppm was detected and assigned to the monopositive cation complex $Au(PEt_3)_2^+$. Further confirmation came from ESIMS analysis. In the spectrum recorded five days after treatment with $AgNO_3$, we found three main signals at 315.1, 333.1 and 433.15 Da assignable respectively to the three species Et_3PAu^+, $Et_3PAu(H_2O)^+$ and $Au(PEt_3)_2^+$. Attributions were validated through theoretical simulation of the various species. Finally, to obtain an additional proof that the ^{31}P NMR signal at 47 ppm corresponds to formation of $Au(PEt_3)_2^+$ species, we synthesized this complex by treatment of Et_3PAuCl with an excess of triethylphosphine. The ^{31}P analysis of the resulting product, confirmed the obtainment of the desired compound and, accordingly, our assignment (S12 for further details on the synthesis and NMR spectrum). A quite different situation was found for the ^{31}P NMR spectra of AF and Et_3PAuCl, for which no significant changes were detected even after very long time intervals. In any case, all three compounds show a large stability in solution even when incubated in presence of NaCl 0.9 % rendering them well suitable for pharmacological testing and application (S11 supporting material).

Next, the antiproliferative properties of the three complexes were measured *in vitro* against HCT8 and HCT116, two representative cell lines of colorectal cancer (CRC). As displayed in Table 1, all three drugs produce potent cytotoxic effects on the selected CRC cell lines with IC_{50} values always falling in the 100-300 nM range. Et_3PAuI is slightly less cytotoxic than the other two gold complexes by a factor~2. In any case, the presence of the thiosugar ligand is not an essential requirement for the cytotoxic action,[13] in line with expectations. In addition, upon considering the close similarity in the measured IC_{50} values, substantial differences in the cellular uptake are unlikely.

Complex	HCT-8	HCT-116
AF	132 ± 16	180 ± 17
Et_3PAuCl	105 ± 11	154 ± 22
Et_3PAuI	260 ± 28	290 ± 36

Table 1. IC_{50} values (nM) determined for Et_3PAuI, AF and Et_3AuP-Cl (24 h incubation). Results are reported as average value for three independent experiments ± SD.

Next, in view of the fact that thioredoxin reductase (TrxR) is a likely important target for AF,[14] we have comparatively quantified the inhibitory power of the three drugs toward this enzyme. Results are summarized in Table 2.

Complex	IC_{50} (nM)
AF	105±17.3
Et_3AuPCl	51.3 ± 8.5
Et_3PAuI	193± 22.2

Table 2. Thioredoxin Reductase activity Assay. IC_{50} values (nM) was determined treating 2U/L of TrxR with aliquots of AF, Et_3AuPCl and Et_3PAuI (from 1μM to 1 nM). Results are reported as average value for three independent experiments ± SD.

It is interesting to note that the obtained IC_{50} values for TrxR inhibition are in line with those obtained for the cytotoxic effects on CRC cell lines (Table 1). This might imply, that the observed cytotoxic effects are somehow related to the ability of these gold complexes to bind and inhibit TrxR. Moreover, results highlight and confirm that Et_3PAuCl is not only the most potent cytotoxic agent, but also the most potent TrxR inhibitory agent of the series, though IC_{50} values for AF and Et_3PAuI still fall in nM range (S18 supporting material for experimental details on TrxR inhibition assay) .

To better characterize the mechanistic aspects of the interactions occurring between these gold compounds and probable biological targets, we have studied their reactions toward the model protein lysozyme (HEWL) and the GG rich oligonucleotide CTACGGTTTCAC (ODN) through ESIMS analysis. Notably, upon replacing the thiosugar ligand with a halide ligand *e.g.* chloride or iodide, we observed a net change in metallodrug reactivity toward HEWL. Indeed, AF interacts appreciably with HEWL, apparently through formation of non-covalent adducts, where the neutral intact drug is bound to the protein (Fig. 3); in contrast, both iodide and chloride derivatives do not react with HEWL even after long incubation times (S15 in supporting material for ESIMS spectra). This different behavior might be ascribed to a crucial role of the thiosugar ligand in forming non-covalent protein adducts.

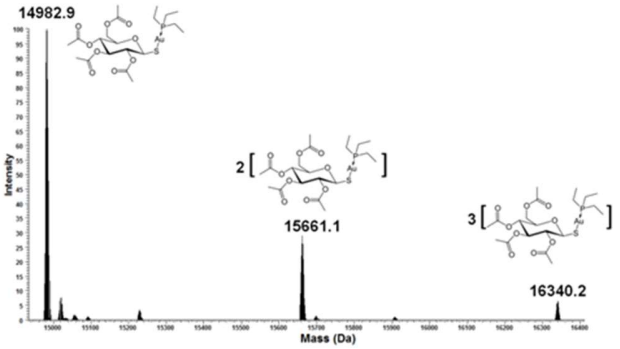

Figure 3. Close-up of deconvoluted ESIMS spectrum (positive mode) of AF incubated 72 h (37° C) with HEWL (10^{-4} M) in ammonium acetate buffer 20 mM pH 6.8 metal to protein ratio (3:1) 3% DMSO.

Conversely, replacement of the thiosugar ligand with halide ligands greatly enhances reactivity toward oligonucleotide. In fact, both Et_3PAuI and Et_3PAuCl coordinate this target through selective release of halide ligands roughly in the same manner (Fig. 4); also, similar amounts of the corresponding adducts are formed.

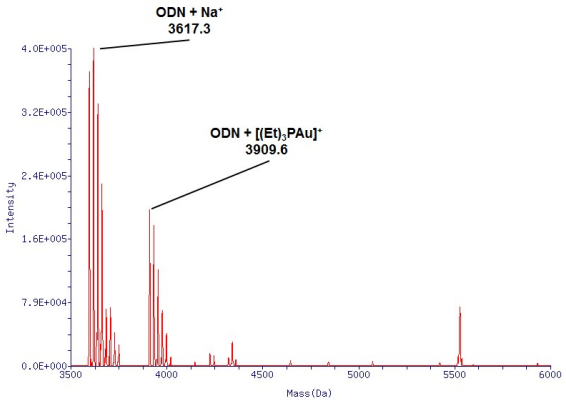

Figure 4. Deconvoluted ESIMS spectrum (negative mode) of Et_3PAuI incubated 72 h (37° C) with ODN (10^{-4} M) in LC-H_2O metal to protein ratio (3:1) 3% DMSO.

In contrast, when performing the same experiment with AF, no adduct formation with ODN was observed; this finding is relevant even considering that AF does not react with double helix DNA as previously reported by Mirabelli and coworkers,[15] and the same kind of reactivity is preserved toward our single strand ODN model. Notably, when AF is incubated with ODN, it only produces a main peak at 923 m/z assignable to the species in Fig. 5, as previously reported (see S16 for spectrum).[16]

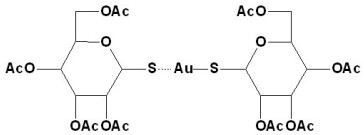

Figure 5. Structure of the main species produced by AF: structure corresponds to the peak at 923 m/z.

Encouraged by the results obtained with ss-ODN, the possible interactions of AF analogues with double stranded DNA (more

precisely calf thymus DNA) were comparatively investigated by the ethidium bromide (EtBr) assay. DNA is first saturated with the EtBr probe producing a fluorescent intercalation complex (while free dye is non-emissive). Then, increasing amounts of the drug are added: a fluorescence decrease indicates progressive EtBr displacement and build-up of drug-DNA interactions.[17,18] We found, indeed, that both Et_3PAuCl and Et_3PAuI produce a net fluorescence decrease while AF does not (see S18 supporting information). The emission decrease is limited, in agreement with the non-intercalative nature of the binding, yet significant. Melting temperatures of drug/DNA mixtures do not vary appreciably from those of DNA alone (changes in the melting temperatures are in the range ± 2 °C in agreement with monodentate coordination of the drug to DNA strands, see S19 supporting material). Conversely, viscosity undergoes significant changes for Et_3PAuCl/DNA and Et_3PAuI/DNA systems compared to the control (Fig. 6, details in supporting material, S20), suggesting that the bound drugs significantly affect the helix flexibility. This strong effect, which is not observed upon AF addition, confirms that the AF-analogues manifest a reactivity significantly different from AF and are able to bind ds-DNA.

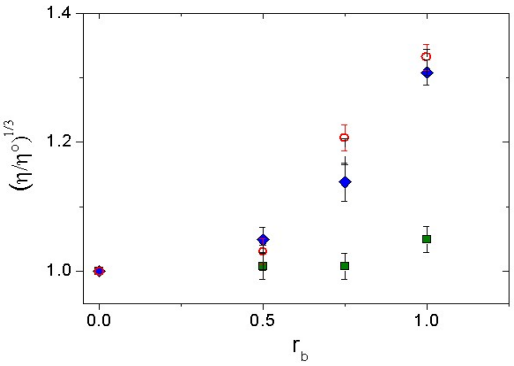

Figure 6. Viscometric plots (25 °C) for Et_3PAuI/DNA (open circles), Et_3PAuCl/DNA (diamonds) and AF/DNA (squares) systems. r_b = [drug]/[DNA] (base pairs), [DNA] = 2.5×10^{-5} M, $\eta/\eta^\circ = (t-t_{solv})/(t_{DNA}-t_{solv})$ where t, t_{solv} and t_{DNA} are the efflux times in the capillary viscometer of the mixture, of the buffer (NaCl 0.1M, NaCacodylate 0.01M, pH 7) and of DNA alone respectively.

In conclusion, we have prepared and characterised here a novel Au(I) complex that is a close analogue of AF featuring the simple replacement of the thiosugar ligand with iodide. Similarly to AF and Et_3PAuCl, Et_3PAuI shows a high stability under physiological-like conditions while manifesting a far greater lipophilic character. Interestingly, Et_3PAuI retains the large cytotoxic effects of AF toward two representative CRC cell lines, with the measured IC_{50} values still falling in the nM range. This implies that the presence of the thiosugar moiety is not mandatory for the pharmacological action. TrxR assay reveals that both Et_3PAuCl and Et_3PAuI retain the potent inhibitory action of AF (nM range), being this consistent with their reported cytotoxic effect. On the other hand, mechanistic differences were highlighted among the three investigated gold complexes with both Et_3PAuI and Et_3PAuCl being unreactive toward HEWL but capable of binding to a standard ss-ODN sequence and to ds-DNA. At variance, AF can bind non covalently to the model protein but does not form coordinative adducts to ssODN and does not bind calf thymus DNA.

In our opinion, the present results are of particular interest for the following reasons:

i) substitution of the thiosugar with iodide and chloride "tunes" the reactivity of these compounds for model biological targets rendering them more selective toward oligo- and polynucleotides. At variance from AF, where the tetra acetate-thiosugar moiety allows formation of non-covalent adducts with lysozyme, this kind of binding is not observed in the case of Et_3PAuI and Et_3PAuCl. Yet, it is worth reminding that even AF is not able to form coordinative bonds to HEWL. Binding of gold(I) metal center to histidine and methionine residues of lysozyme was previously described mainly as naked cation;[19] but this does not occur for AF and its analogues, due to the very high stability of the ligands and their steric hindrance. Furthermore these differences in reactivity toward the model protein HEWL, do not correlate with their strong inhibitory power toward TrxR, being this latter aspect, most probably related with the very high affinity of gold for the selenium donor and thus for the selenocysteine residue;

ii) the enhanced reactivity of the gold(I) center toward the model oligonucleotide and the double helix DNA upon replacement of the thiosugar ligand with halide ligands, may be the result of the greater lability of the gold-halide bond compared to the gold-sulphur bond;[13] the guanine residue of nucleic acids that are highly accessible may "assist" halide detachment. Yet, this augmented reactivity for DNA molecules does not lead to enhanced cytotoxic effects. This might support the view that AF,[20] and also its halido analogues, exert their strong cytotoxic effects mainly through DNA-independent mechanisms

Overall, these findings are of significant interest if one considers that even small differences in biomolecular reactivity may result in large differences in the respective pharmacodynamic and pharmacokinetic profiles. In addition, the far greater lipophilic character of Et_3PAuI might lead to an enhanced bioavailability of the latter drug.

ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of Et_3PAuI; Solution behavior; Analysis after treatment with $AgNO_3$; Stability in physiological like conditions; Synthesis and characterization of $Au(Et_3P)_2^+Cl^-$; Log*P* determination; ESI-MS experiments; X-ray diffraction; Cellular studies (WST-1); Enzyme activity inhibition *in vitro*; Ethidium bromide (EtBr) assay; Melting tests; Viscosimetric measurements.

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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SYNOPSIS TOC. Et_3PAuI has been synthesised, and characterized in comparison with auranofin and Et_3PAuCl. Auranofin analogues retain the potent cytotoxic effects of Auranofin toward CRC cell lines as well as strong TrxR inhibition properties; chemical and mechanistic differences were highlighted..