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Novel anti-cancer agents derived from mucochloric acid

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ABSTRACT

Analogues of a new lead structure were subsequently synthesized by alternative synthetic routes. Various pyrrols, 2(5H)-furanones, bisarylated acrylic acids and 3(2H)-pyridazones were prepared from muco halogen acids in an array of chemical reactions.

The cytotoxicity of these simple butenolides and analogues have been evaluated in tissue culture studies on MAC 13 and MAC 16 murine colon cancer cell lines.

Ketone 5 containing an aromatic moiety only occurred a cytotoxicity *in vitro*, while acyclic bisarylated methacrylic acids 7 showed in addition to a moderate cytotoxicity an inhibition of tumor growth *in vivo* in mice. The xylene derivative 7b displayed at 20mg /kg a 25 % inhibition compared to 27% for the control (5FU).

The acetamido –furanone 8a displayed an IC_{50} of 18, 4 μM for the MAC 13 and MAC16 cell line, respectively and this translated into 26% inhibition of tumour growth in the transplanted MAC 16 cell line in mice.

The unsubstituted pyridazine 9b, had a manifold higher in vitro activity, than the known arylated pyridazone 9a and most interestingly this correlated well with the observed in vivo activity. Pyridazine 9b showed 53% inhibition of tumour growths in vivo in mice at a 50mg/kg dose and less weight loss was observed for this best agent compared to the anti-metabolite 5-FU, which served as standard.

KEYWORDS

Lead structure;
Butenolide;
Anti-cancer agent.

INTRODUCTION

Many cancer patients have metastatic disease at diagnosis and cannot be cured by modern cancer treatment, though there are tumours - for example those of the testes, choriocarcinomas, and Hodgkin's disease - that are now curable even at an advanced stage. Some other tumours- for example in the lung, breast, and prostate- may show considerable benefit from chemotherapy or hormonal manipulation.

Cancer suspect agents like epoxides, aziridines and N-nitroso compounds[1]served as a starting point for the development of anti-cancer agents in the past. Alkylating agents such as Chlorambucil and Cyclophosphamide, which are still in clinical use, contain reactive chlorine atoms as leaving groups[2]. Penicillin acid[3] and Basidalin[4]are butenolide natural products[5]exhibiting antitumour activity in the micromolar range. Butalactin is an antibiotic containing an epoxide side chain[6]. Cis platinum compounds, useful agents in the treatment of

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testicle cancer, display a structural similarity with the dichlorinated derivatives of this research. Penicillin acid is a cytotoxic agent and uracil, as well as tymine, are pyrimidin based nucluotides, whose best known analogue 5-fluorouracil, 5-FU, is a standard anticancer agent (anti-metabolite).

These structurally related furanones (butenolides) and two proposed lead structures with a molecular weight of 210 are outlined in Figure 1.

Initial findings on the synthesis of novel 3,4-dihalogenated 5-substituted 2(5H)-furanones[7,8] were reported and arylated pyridazines were evaluated as potential anticancer agents[9,10].

The synthesis and evaluation of anticancer butenolides from dichloroacetyl chloride and NMF was published in a granted patent showing the principal usefulness of these agents[11].

Here, initially the proposed mechanism of this one pot synthesis is discussed[12] which provided intermediates and lead structures, such as methacrylic acids, 2(5H)-furanones and pyridones. The lead structures were synthesized by alternative chemical routes and these novel analogues were subsequently evaluated *in vitro* in cell culture assays and *in vivo* in mice.

MATERIALS AND METHODS

Chemistry

Chemicals

Muco chloric acid was obtained from Lancaster Ltd (Lancaster, UK). All the other reagents were purchased from Aldrich, UK.

General

Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) was carried out on a Hewlett-Packard 5989B quadrupole instrument connected to an APCI accessory. IR spectra were recorded as KBr discs or in chloroform on a Mattson 3000 FT-IR spectrophotometer. Nuclear Magnetic Resonance spectra were obtained on a Bruker AC 250 instrument with TMS as internal standard.

Tetramic acid chloride 1

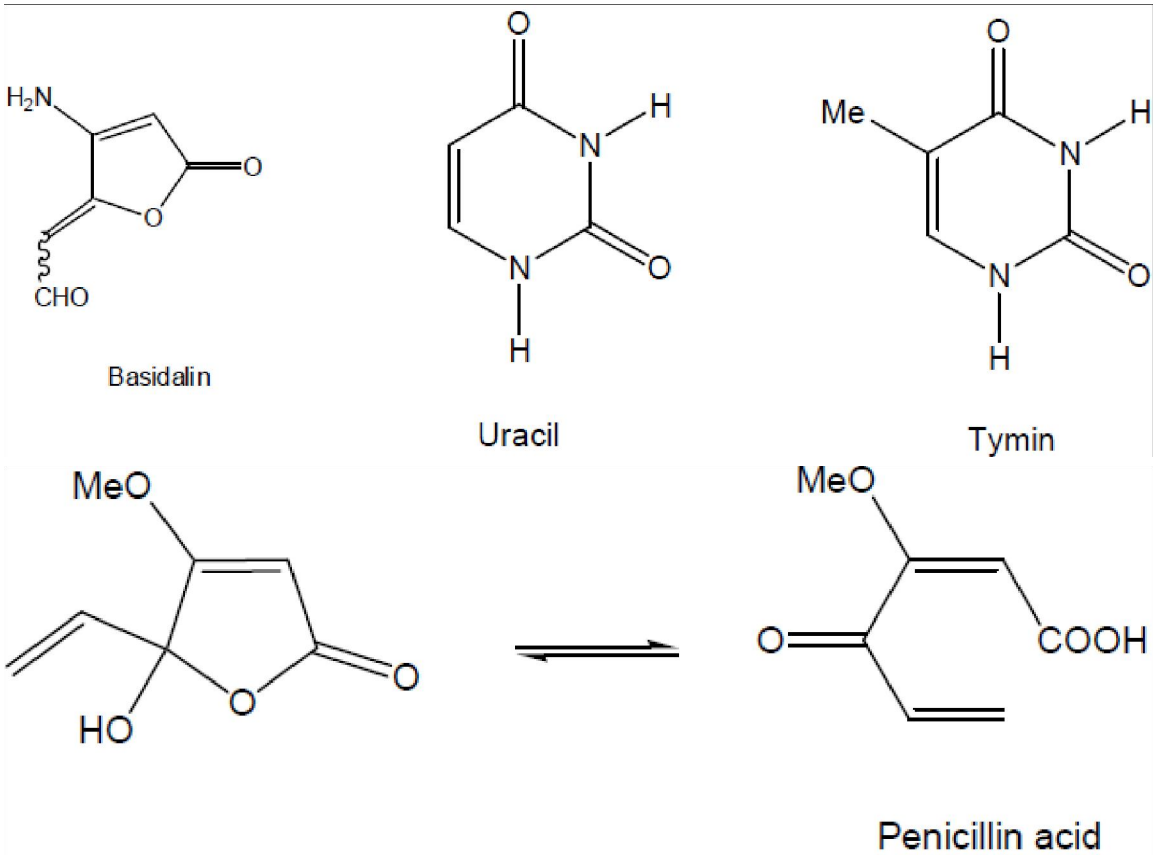

Figure 1 : Overview of chemotherapeutic agents

A solution of ammonia was added dropwise to a solution of the methoxy-furanone (1g, 0.7 mmol) in 9 ml of ether / ethanol (2:1). The solution turned yellow after some h and the desired compound precipitated out over night as slightly brown solid which was purified further by column chromatography with ether / PE (1:1).

Yield: 38 %; APCI+ MS: m/z 197/199; ^{1}H NMR ($CDCl_3$): δ = 9.21(s, 1H, NH), 7.10 (bs, 1H, OH), 6.2 (s, 1H, C6H); ^{13}C NMR ($CDCl_3$): δ = 163.9 (c2), 146.2 (C4), 125.4 (C3), 79.5 (C5); IR: ν =3098m, 2925m, 2855m, 1786s, 1749vs, 1599, 1585, 14441, 1342, 1262, 1157, 1045, 883.

Preparation of 5-Butoxy-3,4-dichlorofuran-2(5H)-one 2

10 g of Muco chloric acid was refluxed with 70 ml of n-butanol and a catalytic amount of sulphuric acid for 48 hours. The reaction was monitored by TLC with ether /petrolether 1:1. After completion the reaction mixture was poured onto ice-cold solution of sodium hydrogen carbonate. The desired compound was extracted with ether petrolether 1:1 and after the removal of the solvent in vacuum the desired compound was obtained as colourless oil.

Yield: 49 %; MS-APCI (+): 226/228(M+1) m/z.; IR ($CHCl_3$) cm^{-1}: 2958, 2873, 2335, 1809, 1637. ^{1}H NMR ($CDCl_3$): δ 5.80 (s, 1H, C<u>H</u>), 3.86-3.59 (m, 2H, OC<u>H</u>$_2$), 1.66-1.35 (m, 4H, OCH$_2$C<u>H</u>$_2$C<u>H</u>$_2$), 0.92 (t, J = 7.3 Hz, 3H, C<u>H</u>$_3$) ppm; ^{13}C NMR ($CDCl_3$) δ 163.24 (<u>C</u>=O), 147.48 (<u>C</u>CH), 124.12 (<u>C</u>C=O), 100.94 (<u>C</u>H), 70.05 (O<u>C</u>H$_2$), 31.15 (OCH$_2$<u>C</u>H$_2$), 18.84 (<u>C</u>H$_2$CH$_3$), 13.59 (<u>C</u>H$_3$) ppm.

Preparation of 3-Chloro-pyrrole-2,5-diones 3

The preparation of 3-Chloro-pyrrole-2,5-dione 3a/ 3b was performed analogue to 3-Bromo-1-methyl-pyrrole-2,5-dione 6.

3-Chloro-pyrrole-2,5-dione 3a

Yield = 11%; M.P: 113-115 ^{o}C; R_f (50% ether / 50% petrol ether) = 0.76

Mol. Weight: 131.5; Mol. Formula: $C_4H_2ClNO_2$; MS (APCI (-)): 130/132 (M-1) m/z

^{1}H NMR ($CDCl_3$) 250 MHz: δ = 7.45-7.95 (br s, NH), 6.71 (s, CH) p.p.m.

^{13}C NMR ($CDCl_3$) 250 MHz: δ = 168.8 (CH-<u>C</u>=O), 161.3 (C-Cl-<u>C</u>=O), 140.6 (C-Cl), 128.7 (CH) p.p.m. IR (KBr-disc) υ max: 3481, 3243, 3107, 2705, 1847, 1774, 1712, 1612, 1595, 1338, 1239, 1137, 1052, 1038, 879, 735, 664, 557, 483 cm^{-1}

3-Chloro-1-methyl-pyrrole-2,5-dione 3b

Yield = 22%; M.P: 88-90 ^{o}C; R_f (50% ether / 50% petrol ether) = 0.81; Mol. Weight: 145.5;Mol. Formula: $C_5H_4ClNO_2$; MS (APCI (+)): 146/148 (M+1) m/z

^{1}H NMR ($CDCl_3$) 250 MHz: δ = 6.65 (s, CH), 3.11 (s, CH_3) p.p.m.; ^{13}C NMR ($CDCl_3$) 250 MHz: δ = 167.8 (CH-<u>C</u>=O), 165.7 (CCl-<u>C</u>=O), 140.9 (C-Cl), 126.8 (CH), 24.4 (CH_3) p.p.m.; IR (KBr-disc) υ max: 3470, 3099, 2956, 1826, 1780, 1723, 1703, 1600, 1440, 1386, 1246, 980, 875, 852, 709 cm^{-1}

3,4-Dichloro-5-(4-methoxyphenyl)furan-2(5H)-one 4

Phosphorus pentoxide (15.7 g) was added to phosphoric acid (92.8 g) and to this mixture finely powdered muco chloric acid (17.1g) followed by anisole (10.8g) was added. This mixture was reacted over night at ambient temperature. For work up it was poured onto ice with 150 ml of hydrochloric acid. The mixture was extracted with ether and the combined organic phases were dried with sodium sulphate. The solvent was evaporated off in vacuum to give a yellow oil.

Yield: 76%. MS-APCI (+): 259/260 m/z; IR ($CHCl_3$) cm^{-1}: 3542, 3027, 1797, 1778, 1632, 1604, 1113. ^{1}H NMR ($CDCl_3$): δ 7.33-7.12 (m, 2H, *m-Ar*-<u>H</u>), 6.96-6.90 (m, 2H, *o-Ar*-<u>H</u>), 5.79 (s, 1H, C<u>H</u>), 3.80 (s, 3H, *p*-OC<u>H</u>$_3$) ppm. ^{13}C-NMR ($CDCl_3$): δ 162.63 (<u>C</u>=O), 147.04 (CH<u>C</u>Cl), 130.56, 128.40, 127.18, 121.02, 120.11, 82.21(<u>C</u>H), 59.23 (*p*-OC<u>H</u>$_3$) ppm.

3,4-dichloro-5-(2-oxo-2-phenylethyl]furan-2(5H)-one 5

Muco chloric acid (21.0 g, 0.125 mol) and acetophenone (0.135mol) were each dissolved in methanol (200 ml) and cooled to 0^{o}C. A solution of NaOH (8.0 g in 70 ml water, 2.5 M) was added slowly under stirring at 0-5 ^{o}C. After the addition of NaOH, the mixture was allowed to stand at RT for 3 h. The brown mixture was poured into ice water, containing an excess of conc. HCl and allowed to stand for 45 min. A

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yellow oily liquid/solid was decanted and washed with water to give a crude yellow product. It was recrystallized from hot ethanol to give the pure compounds as a white powder (mp 117-118°C).

Yield: 72%, IR (KBr) cm^{-1}: 3010, 1773, 1683, 1648, 1210, 1033, 767, 692. ^1H NMR ($CDCl_3$) δ 3.35-3.61 (m, CH_2), 5.69-5.73 (dd, CH, J= 3.6 Hz), 7.45-7.52 (t, Ar-2H, J= 7.8, 7.2 Hz), 7.56-7.65 (tt, Ar-H, J= 7.4, 7.3 Hz), 7.91-7.95 (d, Ar-2H, J= 7.1 Hz) ppm. ^{13}C NMR ($CDCl_3$) δ 40.0 (CH_2), 78.0 (CH), 121.4 (C-Cl), 128.1 (2xC), 134.1, 135.7 (2xC) (Ar-C), 151.9 (C-Cl), 164.8 169.9, 193.7 (C=O) ppm.

3-Bromo-1-methyl-pyrrole-2,5-dione 6

Method: Dry mucobromic acid (15.0 g, 88.8 mmol) and 3 equivalents of N-methylformamide (287 mmol) were refluxed in toluene (100 ml) with 1% conc. H_2SO_4. TLC was used to monitor the reaction progress (ether/ petrol ether). After 8 hours, the liquid phase of the mixture was poured off and silicagel was added until a light brown fine powder was obtained. The mixture was extracted using solid extraction column chromatography (20% ether/ 80% petrol ether) and remaining solvent was evaporated off under vacuum to give a pure crystalline compound.

Yield = 61%; M.P: 90-92 °C; R_f (50% ether / 50% petrol ether) = 0.72; MW: 190; Molecular Formula: $C_5H_4BrNO_2$, MS (APCI(+)): 190/192 (M+1), 222/224 (M+MeOH) m/z; ^1H NMR ($CDCl_3$) 250 MHz: δ = 6.89 (s, CH), 3.11 (s, CH_3) p.p.m.

^{13}C NMR ($CDCl_3$) 250 MHz: δ = 168.6 (CH-<u>C</u>=O), 165.6 (C-Br-<u>C</u>=O), 131.9 (CH), 129.4 (C-Br), 25.4 (CH) p.p.m. IR (KBr-disc) υ max: 3464, 3105, 2947, 1778, 1714, 1707, 1585, 1429, 1371, 1287, 1139, 1102, 997, 962, 867, 817, 796, 756, 698 cm^{-1}

2,3-Dichloro-4,4-bis(3,4-dimethylphenyl)but-2-enoic acid 7a

8.19 g (48.5 mmol) mucochloric acid was dissolved in 200 ml of the appropriate xylene. 10 g powdered aluminium chloride was added slowly to the resultant solution, capped with a drying tube and stirred for 72 hrs. The crude product was poured into a beaker containing 125 g ice and 38 g conc. HCl. The organic phase separated from the aqueous phase by extraction using toluene. The resulting solution was dried over magnesium sulphate and excess solvent was removed under vacuum, before the product was dried under vacuum in a dedicator.

Yield: 69 %; $C_{20}H_{20}Cl_2O_2$, MW: 363.28. APCI+ M/S: 326/327; ^1H-NMR ($CDCl_3$): δ = 7.18-6.98 (m, 6H, aryl-<u>H</u>), 6.59 (s, 1H, C<u>H</u>), 2.34-2.21 (m, 12H, C<u>H</u>$_3$) ppm (carboxylic acid group not detectable). ^{13}C-NMR ($CDCl_3$): δ = 166.17 (<u>C</u>OOH), 153.67 (Cl<u>C</u>CH), 136.87 & 136.57 & 135.62 & 130.28 & 129.54 & 126.53 (aryl-<u>C</u>), 121.97 (<u>C</u>COOH), 50.02 (<u>C</u>H), 19.84 (aryl-<u>C</u>H$_3$), 19.38 (aryl-<u>C</u>H$_3$) ppm.IR: ν = 2971, 2947, 2921, 2652, 2510, 1730, 1695 cm^{-1}.

2,3-Dichloro-4,4-bis(2,4-dimethylphenyl)but-2-enoic acid 7b

Yield = 85%; $C_{20}H_{20}Cl_2O_2$, Molecular Weight: 363.28.; APCI+ M/S: 326/327. EI MS: m/z = 2362/ 364 (M+), 326, 291, 247, 232, 215. IR: ν = 2969, 2925, 2865, 2649, 1730, 1693 cm^{-1}. ^1H-NMR ($CDCl_3$): δ = 7.01-6.88 (m, 6H, aryl-<u>H</u>), 6.62 (s, 1H, C<u>H</u>), 2.31 (s, 6H, *o*-C<u>H</u>$_3$), 2.11 (s, 6H, *p*-C<u>H</u>$_3$) ppm (carboxylic acid group not detectable).

^{13}C-NMR ($CDCl_3$): δ = 166.41 (<u>C</u>OOH), 155.23 (Cl<u>C</u>CH), 136.83 & 136.35 & 135.62 & 131.21 & 128.29 &126.63 (aryl-<u>C</u>), 122.36 (<u>C</u>COOH), 49.02 (<u>C</u>H), 20.95 (aryl-<u>C</u>H$_3$), 19.22 (aryl-<u>C</u>H$_3$) ppm.

Preparation of Dichloro-5-oxo-2,5-dihydro-furan-2-yl-acetamides 8

Method: Dry mucochloric acid (15.0 g, 88.8 mmol) and 3 equivalents of N-methylformamide/acetamide (287 mmol) were refluxed in toluene (100 ml) with 0.5% conc. H_2SO_4. TLC was used to monitor the reaction progress (ether/petrol ether). After 12 hours, silicagel was added until a light brown fine powder was obtained. The mixture was extracted using solid extraction column chromatography (20% ether/ 80% petrol ether) and remaining solvent was evaporated off under vacuum to give a pure crystalline compound.

Dichloro-5-oxo-2,5-dihydro-furan-2-yl)-N-methyl-acetamide 8a

Yield = 31%; M.P: 119-121 °C; Rf (10% methanol / 90% ether) = 0.37

MW: 224.0; Molecular Formula: $C_7H_7NO_3Cl_2$, MS (APCI(+)): 182/184/186 (M+1), 224/226/228 (M+) m/z; ^1H NMR ($CDCl_3$) 250 MHz: δ = 6.48 (s, CH),

2.69 (s, N-CH_3), 2.44 (s, CO-CH_3) p.p.m. ^{13}C NMR ($CDCl_3$) 250 MHz: δ = 172.4 (N-CO), 163.5 (CO), 148.1 (CH-C-Cl), 124.3 (C-Cl), 83.4 (CH), 29.1 (N-CH_3), 22.1 (CO-CH_3) p.p.m.

IR (KBr-disc) υ max: 3436, 2949, 2843, 1787, 1663, 1631, 1397, 1314, 988, 745 cm^{-1}

Crystal Data - (sample recrystallised from methanol):

$C_7H_7Cl_2NO_3$	
M_r = 224.04	
T = 293(2) K	
Tabular	V = 2772.4(5) Å^3
0.30 x 0.10 x 0.05 mm	Z = 2
Colourless	D_x = 1.604 Mg/m^{-3}
Mo Kα radiation: λ = 0.71073 Å	D_m not measured
Triclinic	R [$F^2 > 2\sigma(F^2)$] = 0.0615
P-1	R(F^2) = 0.1289
a = 6.4718(15) Å	2138 reflections
b = 8.3329(9) Å	120 parameters
c = 9.708(2) Å	
α = 93.049(17) º	
β = 94.791(12) º	
γ = 107.651(19) º	

Selected geometric parameters (Å, º)

Cl(7)-C(2)	1.698(7)	O(5)-C(4)	1.467(9)
Cl(8)-C(3)	1.671(7)	C(2)-C(3)	1.327(9)
O(6)-C(1)	1.175(8)	N(9)-C(4)	1.408(9)
O(13)-C(11)	1.211(8)	N(9)-C(10)	1.474(8)
O(5)-C(1)	1.378(9)	O(13)-C(11)	1.211(8)
C(1)-C(2)-Cl(7)	120.6(5)		
C(4)-C(3)-Cl(8)	121.6(5)	C(2)-C(3)-Cl(8)	128.3(6)
O(6)-C(1)-C(2)	131.0(7)	C(4)-N(9)-C(10)	118.8(5)
O(6)-C(1)-O(5)	122.2(7)	O(13)-C(11)-N(9)	120.2(7)

^{13}C NMR ($CDCl_3$) 300 K δ: (Isomers)(28.7, 29.9, 30.17), (50.3, 51.1, 53.0) (CH_3), 61.6, 63.1 (C(CH_3)$_3$), 106.4 (CH), 148.8 (C-Cl), 160.8 (C-Cl-CO), 180.4 (CO-O), 192.9 (C=O) p.p.m.

Preparation of 4,5-Dichloro-3-pyridazones 9

4,5-Dichloro-2-p-methoxy-phenyl-3-pyridazone 9a

A solution of muco chloric acid (2.1g, 0.012 mol) in methanol/water (1:1, 10 ml) was stirred at room temperature. To this solution a mixture of the p-methoxyphenyl-hydrazine hydrochloride (0.016 mol) in methanol/water (1:1, 10 ml) was added drop wise and stirred at room temperature. The uncyclized hydrazone intermediate was obtained as an orange-brown precipitate, which was collected after 3 h by filtration. Glacial acetic acid was slowly added at 100-110ºC to the hydrazone intermediate and the mixture was subsequently refluxed for 20 min. The solution was diluted with water and cooled on ice. On cooling the pyridazone adduct was obtained as a beige, coloured powder. After recrystallization from 90% ethanol, a white powder was obtained (mp 176-177°C).

Yield: 39%. MS-APCI(+): 273/275 (M+1) m/z; IR (KBr) cm^{-1}: 3049, 2924, 1818, 1655, 1572, 1241, 1136, 943, 807.

^{1}HNMR ($CDCl_3$) δ 3.80 (s, 3H, OMe); 7.64 + 7.10 (d, 2H+2H, Ar-H), 8.26 (s, 1H, -CH) ppm; ^{13}CNMR ($CDCl_3$) δ 60.34 (*p*-OCH_3) 126.4, 129.3, 134.3, 136.1 (Ar-C), 136.9, 137.1, 139.6, 154.2 (C=O) ppm.

3-4-dichloro-5-oxo-2,5-dihydrofuran-yl(methyl)-formamide 8b

Yield: 20.5 %; R_f (10% MeOH/ether)= 0.53; Mol. Formula: $C_6H_5Cl_2NO_3$. MW: 210; IR (KBr-disc) υ max: 2961, 1806, 1701, 1408, 1299, 1030, 913, 747 cm^{-1}.MS (APCI(+)): 210 (M+1) m/z. ^{1}H NMR (DMSO-d_6) 300 K δ: (Isomers) 2.60, 2.84 (s, CH_3), 6.22, 6.80 (s, CH), 8.37, 8.52 (s, COH) p.p.m.^{13}C NMR (DMSO-d_6) 300 K δ: (Isomers) 24.4, 28.3 (CH_3), 81.5, 88.6 (CH), 124.0, 124.9 (C-Cl), 146.3, 147.1 (C-Cl-CO), 161.8, 162.5 (CO-O), 163.8, 167.4 (C=O) p.p.m.

tert-butyl(3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl)-formamide 8c

Yield: 11.9 %; R_f (10% MeOH/ether)= 0.61; IR (KBr-disc) υ max: 3279, 2971, 1679, 1614, 1392, 1346, 1266, 1195, 1006 cm^{-1}. MW: 252.1. MS (APCI(+)): 253 (M+1), 162, 163, 164 (M+) m/z. ^{1}H NMR ($CDCl_3$) 300 K δ: (Isomers) 1.25-1.32 (m, CH_3, 9H), 7.27, 8.14 (s, CH), 7.82, 8.89 (s, COH) p.p.m.

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4,5-Dichloro-2-pyridazin-3(2H)-one 9b

A solution of muco chloric acid (2.1g, 0.012 mol) in methanol/water (1:1, 10 ml) was stirred at room temperature. To this solution semicarbazide hydrochloride (1.81 g, 0.016 mol) in methanol/water (1:1, 10 ml) was added drop wise and stirred 20 min at room temperature. To the resultant precipitate 10 ml of acetic cid was added and refluxed until the development of carbondioxide ceased (30 min). The solution was diluted with water and cooled. On cooling the pyridazone adduct was obtained as an off white powder. After recrystallization from 90% ethanol, a white crystalline powder was obtained (mp 202-203°C).

Yield: 73%. MS-APCI(+): 276/278 (M+1) m/z; IR (KBr) cm^{-1}: 3215, 3030, 1815, 1633, 1216, 1142, 1035, 816. ^{1}HNMR ($CDCl_3$) δ 7.78–7.74 (m, 4H, Ar-H), 8.26 (s, 1H, -CH) ppm; ^{13}CNMR ($CDCl_3$) δ 128.4, 134.6, 134.8, 135.3, 136.9, 137.3, 143.6 (N-C-Ar), 159.6 (C=O) ppm.

Biological evaluation

In vitro cytotoxicty

The cytotoxicity was determined against the murine carcinoma cell lines (MAC13 and MAC16) using the standard MTT assay [13].

The culture media used was RPMI 1640 containing hepes, glutamine, antibiotics and supplemented with 10% fetal calf serum for MAC13 cells and 5% fetal calf serum for MAC16 cells. Cells were counted by the trypan blue exclusion method using a plastic Kova counting chamber. The MAC 13 and MAC16 cells were suspended in appropriate volumes of media and were seeded at 0.5 x 10^4 and 2 x 10^4 cells / 200 µl respectively in flat-bottomed 96 well plates. The test compounds were dissolved in dimethyl sulphoxide (DMSO) to give stock solutions of 100 mM. Dilution series from 10^{-4} M to 10^{-9} were made so that each compound was tested at six concentrations and in triplicate.

5-Fluoro-uridine (5-FU), a known anticancer agent, was used as a control and tested in the 20-0.02 µM range. Plates were then incubated at 37°C, 5% CO_2 for three days. Compounds were tested on at least two separate occasions. On day three 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (7.5mg MTT /ml of PBS) was added to each well and plates were allowed to incubate for a further 2 h. 120 µl of culture supernatant was carefully removed from each well and 100 l of acidified i-propanol containing 10% Triton-X100 was then added to each well. Plates were agitated for 10 minutes at 800 rpm on a plate shaker. Following this solubilisation step all plates were then read, within 15 minutes, on an Anthos AW200 plate reader at 540 nm with a reference wavelength of 590 nm.

In vivo experiments in mice - assessment of anti-tumour inhibition

Pure strain NMRI mice aged between 6 and 8 weeks from our inbred colony were used for transplanting MAC (murine colon cancer) tumours. Animals were fed on RM3E diet (Lillco-England) and water *ad libitum*. Approximately 2 mm cubes containing 2x 10^5 cells of MAC 16 tumour fragments were transplanted subcutaneously in the inguinal region via a trocar in a volume of 0.2 ml. Tumour bearing mice were randomised in groups of 7 animals per group and the treatment was started 10 days after transplantation. The test compounds were administered in propylene glycol. The effect of chemotherapy was assessed 20 days after transplantation. Mice were killed after 10 days of drug treatment and the effects were measured by the differences in tumour weight as expressed: % [inhibition] = Treated weight / control weight x 100

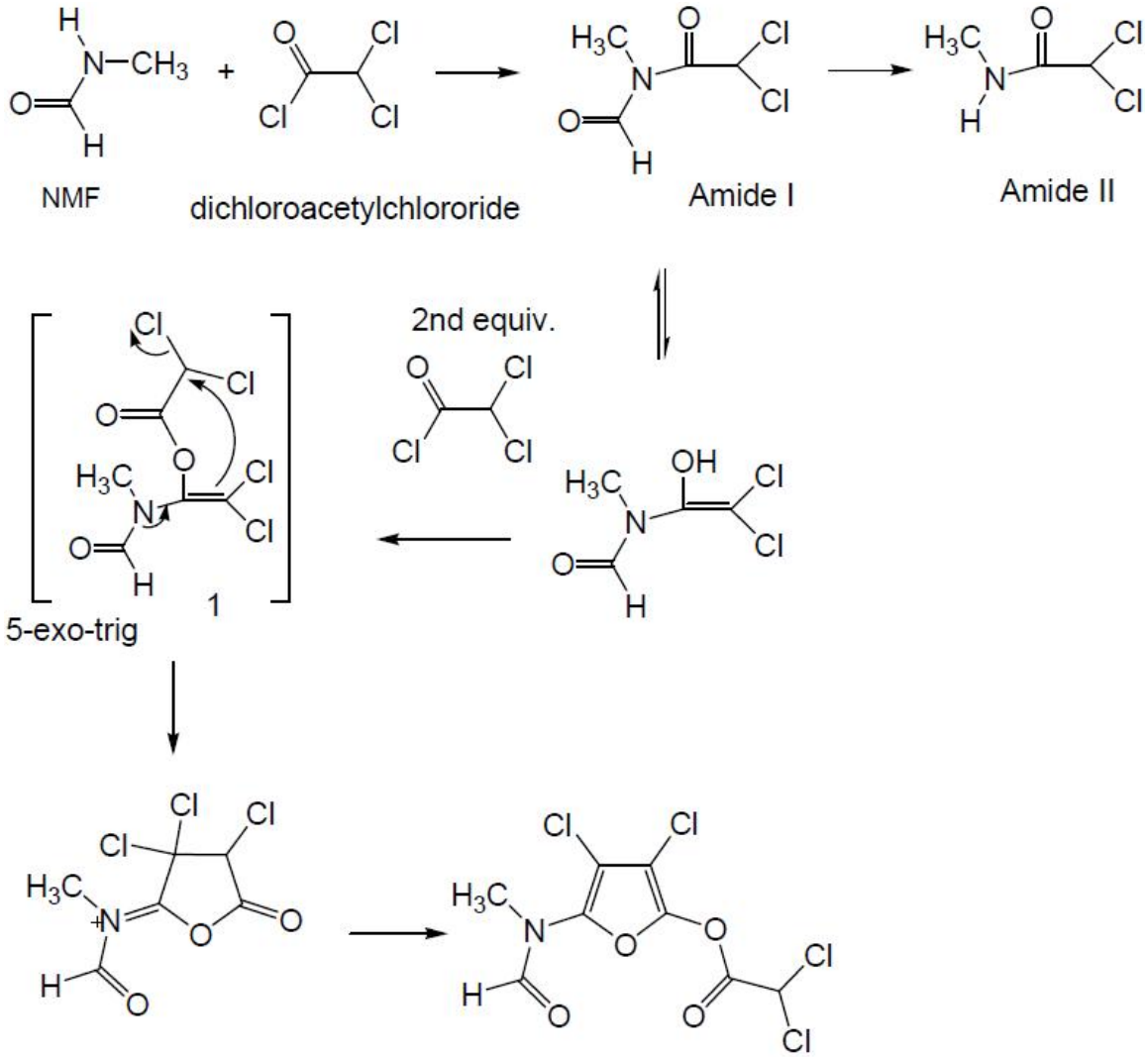
The body weight changes were recorded additionally. The procedure was approved by the home office and the bioethics committee of Aston University.

The results are expressed as mean ± S.D. and the data were subjected to repeated measures of the one-way analysis of variance (ANOWA). If the probability level (p-value) is less than 0.05, a statistical significance was attained.

RESULTS AND DISCUSSION

Chemistry- mechanism of the proposed lead structures

NMF and dichloroacetyl chloride formed the expected amide I, loss of CO resulted in amide II. The mechanism for the one pot synthesis of multiply-substituted furans is proposed as followed and required reflux conditions and excess of dichloroacetyl chloride (Scheme 1).



Scheme1 : Formation of lead structures from DMF and excess of DCACl under reflux, part 1

Mechanistically, the enol form of the amide I reacted with a second molecule of dichloroacetylchloride. This acyclic intermediate containing an electrophilic α-position and a nucleophilic ene-amine substructure, formed the lactone ring system in a 5-exo-trig reaction. Subsequent reaction with a third molecule of dichloroacetyl chloride gave the desired furan.

The pure furan was converted with hydrogencarbonate into the methacrylic derivative and the amido furanone with a molecular weight of 210.

In presence of base the methacrylic acid derivative cyclised into the 7-membered ring intermediate, which reacted *in situ* in a retro-Bayer-Villiger rearrangement into the desired dihydropyridone. The oxygen atom was transferred to the carboxylic acid.

For the furan synthesis the addition of NMF to the dichloroacetyl chloride was essential.

Tautomeres of the target molecule are outlined in figure 2. The structural similarities of the lead structure with the nucleotides, outlined in figure 1, are recognized in addition to the alkylating properties of the agents.

3,4-dichloro-6-hydroxy-1-methyl-1,6-dihydropyridine-2,5-dione

Overall, at least 2 lead structures with a molecular weight of 210, served as starting point of the synthesis of structurally related analogues.

Analogue synthesis

The overall starting materials are muco-halogen acids, which are commercially available form furfural. Furfural is cheaper than DCM, dichloromethane, and it is available from biomass in unlimited quantities. Therefore, any synthetic application of furfural is an example of green chemistry, in which petrochemical intermedi-

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ates, were replaced by raw materials from biomass. Hay, the remains of rice production and other by-products from food production can be converted by acid, such as hydrochloric acid into furfural.

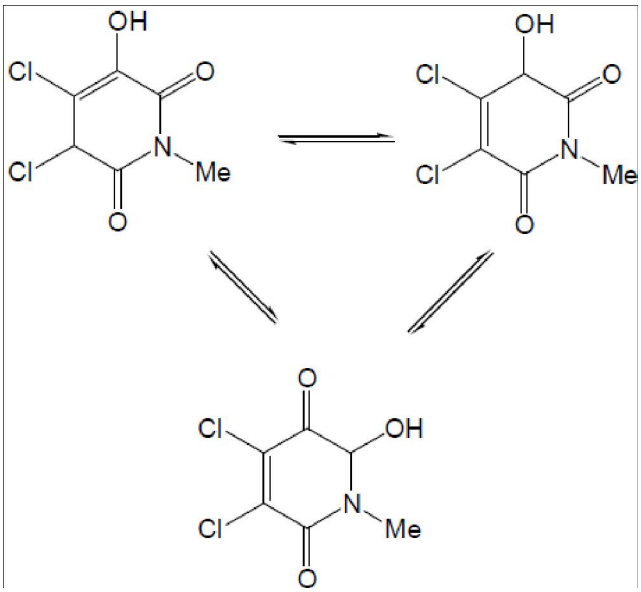
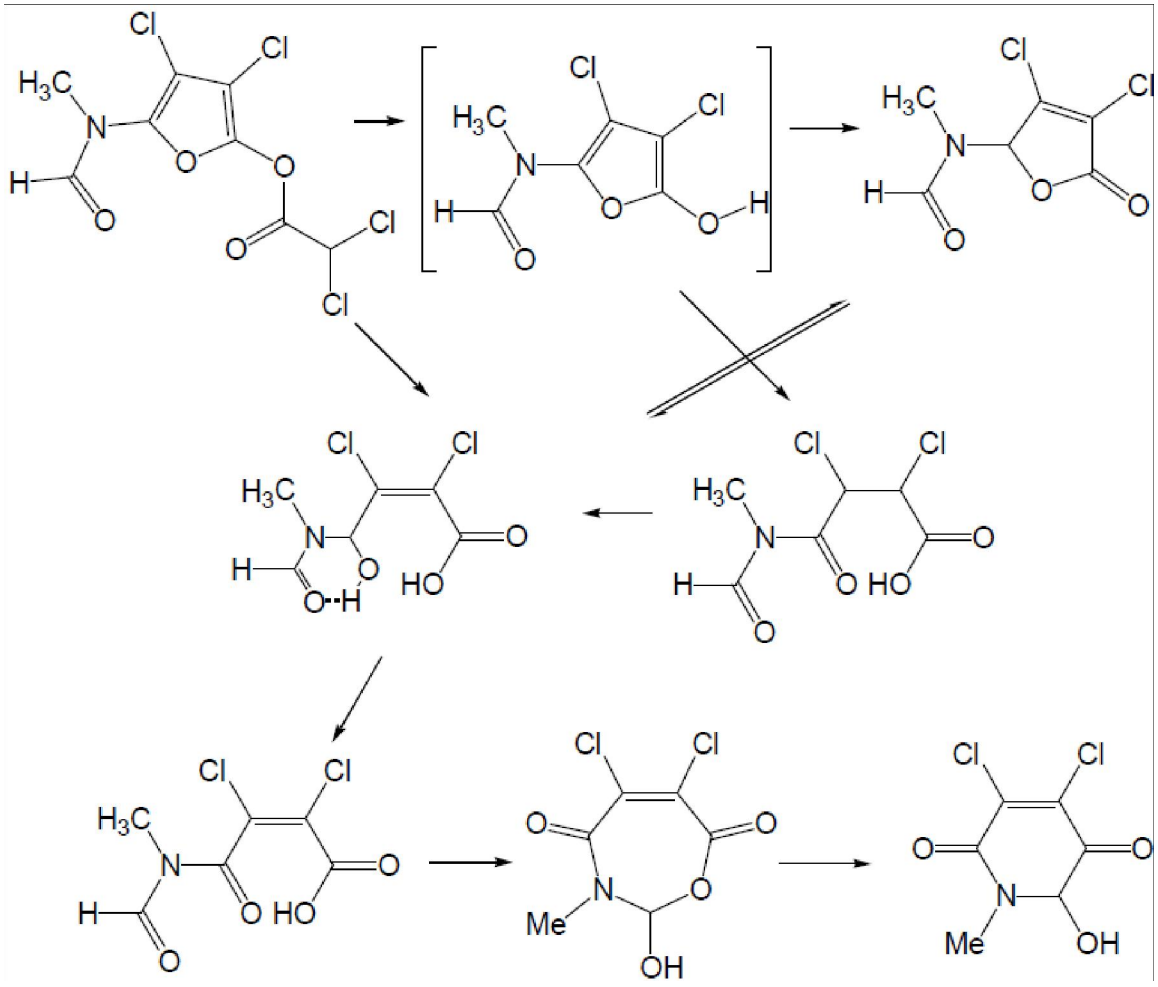


Figure 2 : Tautomers: Main or only tautomeric form

The tetramic acid 1 is the aza - analogue of mucochloric acid, and a direct conversion of mucochloric acid with ammonia resulted in a complex mixture. The protection of the aldehyde of as methoxy semi-acetale was essential and gave the lactone 1 in good yield as solid material.

The pseudoester 2 was synthesised by refluxing muco chloric acid in n-butanol in presence of a catalytic amount of sulphuric acid.

A series of pseudoesters and pseudoanhydrides were synthesised as described in Lattmann et al 2001, in which the hydroxy-tautomeric form of muco chloric acid was reacted. The chloro-pyrrol 3a and 3b were formed from mucochloric acid and formamide / acetamide under acetic conditions in low yields as a by-product of the synthesis of the amide series 8.



Scheme 2 : Design of lead structures. Base treatment at RT, part 2

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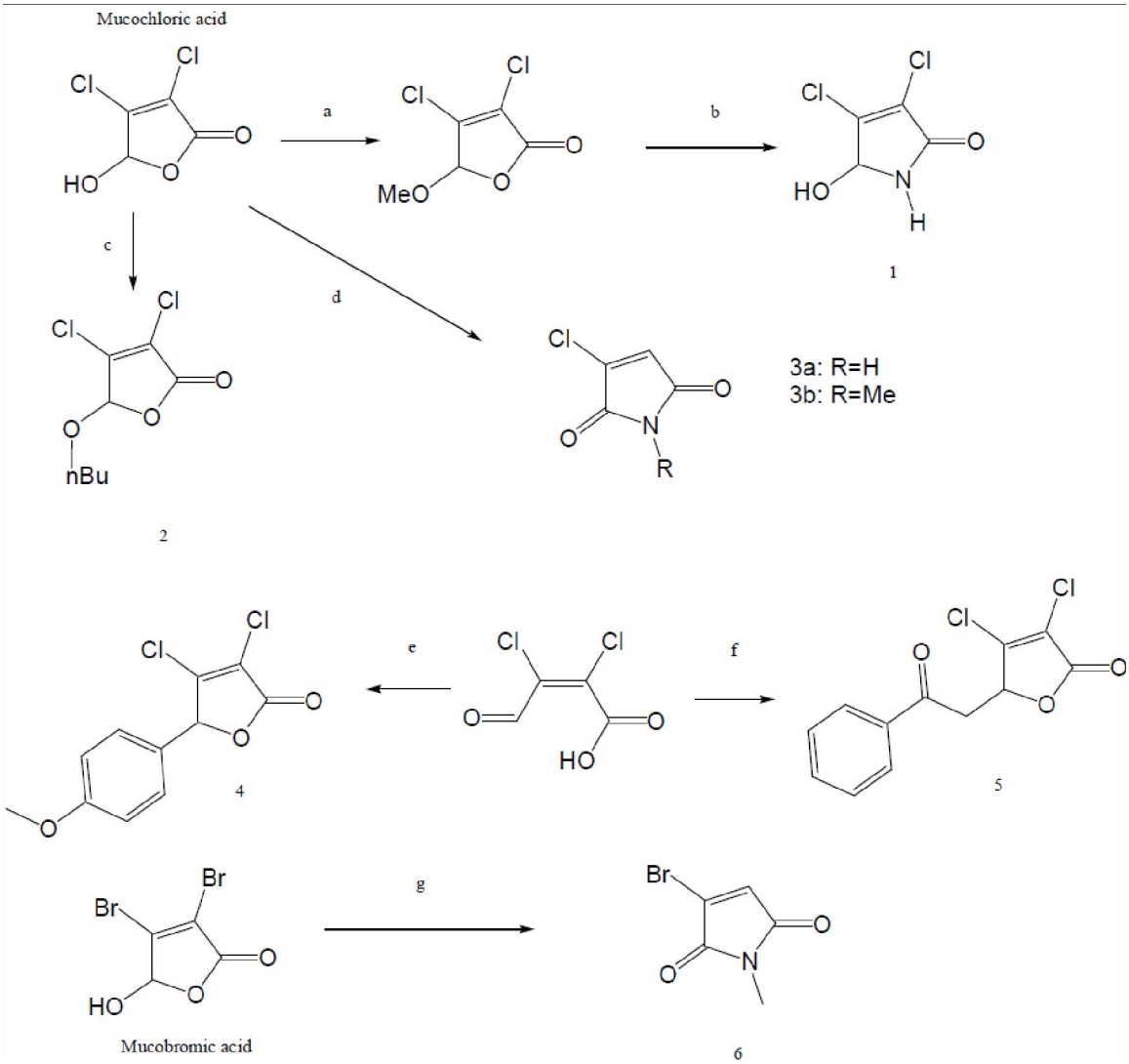
The free tautomeric aldehyde form (scheme 3) of muco chloric acid [14] was reacted in aldol like reactions (entry 5) and electrophilic aromatic substitutions reactions (entry 4) via ring opened hydroxy-intermediates into 5-substituted 2(5H)-furanones.

Mucochloric acid was reacted with anisol in presence of a Lewis acid into the furanone 4 [15] using a mixture of phosphorus pentoxide and phosphoric acid at ambient temperature. Aluminium chloride as Lewis acid furnished tar like material.

Acetophenone furnished under alkaline conditions at ambient temperature, via a 4-hydroxy acrylic acid intermediate, ketone 5 in an excellent yield as a solid material [16].

Mucobromic acid was converted using formamide under reflux conditions with toluene into the bromopyrrol 6. For the mucobromic acid the outlined pyrrolone derivative is the major reaction product, which was formed in a good yield.

Dichlorinated bis-arylated acrylic acids 7a and 7b were derived from mucochloric acid in excess of xylene in presence of aluminum chloride as Lewis acid in very good yields. Toluene formed a mixture of the bisarylated acid, analog to entry 7 and the 5-arylfuranone, analog to entry 4.



Scheme 3 : Synthesis of analogues from mucohalogen acids, part 1. a) MeOH, reflux, cat. H_2SO_4, b) ammonia, RT, c) n-butanol, reflux, cat H_2SO_4, d) NMF, reflux, cat H_2SO_4, e) anisol, RT, H_3PO_4, P_4O_{10}, f) acetophenone, NaOH, ice, g) NMF, reflux, toluene

A series of amido-furanones 8a-8c were formed from mucochloric acid, by refluxing the parent amide in toluene. Thus, the analogue 8b of the original form-amido furanone structure with the molecular weight of 210,

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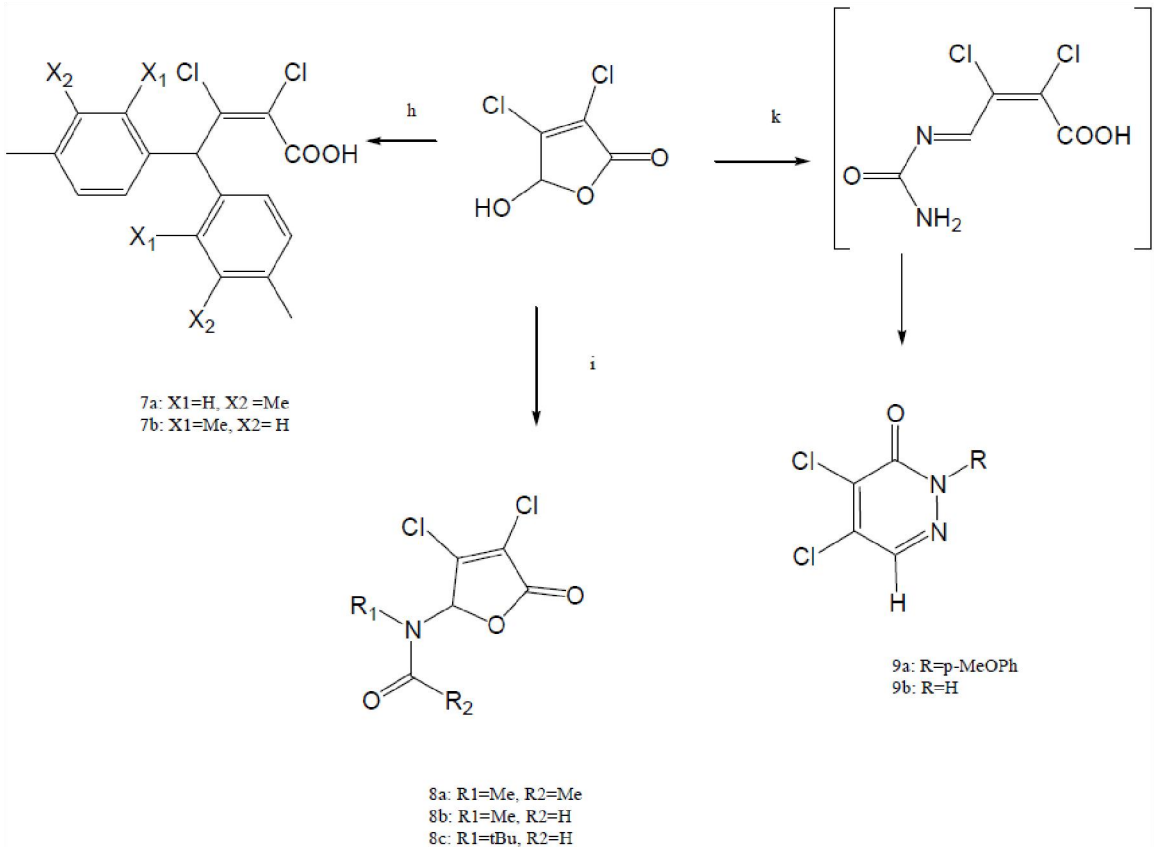
was synthesized by an alternative route. Purification of the amido-furanone series 8 was performed by column chromatography, which was essential in order to remove the pyrrol-diones byproducts 3.

The desired pyridazones 9a-9b were synthesised in two steps from muco chloric acid[17]. The parent p-methoxy-phenylhydrazine and semicarbazide were used to prepare them as crystalline materials in high yields. The substituted hydrazine /semicarbazide gave the hydrazone/semicarbazone intermediate of the dichloro acrylic acid derivative in methanol at ambient temperature. Subsequent cyclisation of these reaction intermediates in acetic acid furnished the crystalline pyridazones 9a-9b. The semicarbazide method was superior to the previous synthesis of 9b using hydrazine, which gave the desired product only in low yield [18].



Scheme 4 : Synthesis of analogues from mucochloric acid, part 2; h) xylene, $AlCl_3$, RT; i) amide, toluene, reflux; k) semicarbazide/ hydrazine, MeOH; reflux acetic acid

Pharmacology, *in vitro* - *in vivo* - evaluation

The *in vitro* screening results, using the standard colorimetric MTT assay for the selected murine colon adenocarcinoma MAC 13 and the MAC 16 cell lines, are outlined in TABLE 1.

The aza - analogue of the *in vitro* inactive mucochloric acid showed an interesting selectivity on the chemo-resistant MAC 16 cell line and represents an interesting lead structure, which is currently being investigated.

The pseudo halogen acids, such as mucochloric acid showed *in vitro* activity IC_{50} >100 μM. The pseudo-ester [19]series have shown a maximum cytotoxicity for agent 2 containing a butyl- substituent in the 5-position. The 5-butoxyfuranone 2 was used to compare the anti-cancer activity of previous work[10]and this new series of antineoplastic agents.

The pyrroldione 3a and 3b showed an interesting *in vitro* cytotoxicity in the micromolar range, but also an acute toxicity *in vivo* in mice, which was assessed as percentage weight loss. The weight loss was found above 10% of lean body mass within 3 days at a dose of 20 mg/kg.

The arylated furanone 4 is an interesting starting point for a new development programme as its acute toxicity is lost, when kept over the weekend in an

aquous formulation. Ketone 5 was found biologically moderately active *in vitro* and the advantage of this solid molecule, compared with the ester series are its good physical properties. The ketone 5 contained an aromatic moiety, which was supposed to intercalate into the DNA. However, this white stable crystalline molecule did not show a significant inhibition of tumour growth *in vivo* compared to the untreated group.

TABLE 1 : In vitro activity / cytotoxicity of synthesized analogues

Entry	Structure	Yield (%)	MAC 13 IC_{50} [μM]	MAC 16 IC_{50} [μM]
	Muco chloric acid		>100	>100
	Muco bromic acid		>100	>100
1	Tetramic acid-Cl	38	13±0.4	8±0.2
2	n-butylester	49	3±0.4	3±0.2
3a	Cl-Pyrrol	11	8±0.6	6±0.4
3b	Cl-Pyrrol	22	12±2	15±3
4	5-Arylated furanone	76	40±6	50±6
5	Ketone	72	30±2	40±2
6	Br-Pyrrol	61	3±0.4	4±0.4
7a	Acrylic acid	69	30±3	50±4
7b	Acrylic acid	85	50±3	70±5
8a	Amido-furanone	31	18±3	4±2
8b	Amido-furanone	20	17±1	11±1
8c	Amido-furanone	12	22±2	19±2
9a	Pyridazine	39	17±2	35±3
9b	Pyridazine	73	7±0.6	5±0.4

TABLE 2 : *In vivo* data of selected molecules against MAC 16 cell lines

Entry	Administration (mg/kg)	Tumor Inhibition (%)	Body-Weight (g)	Treatment (days)
Water	0	0	+0.1±0.05	1 - 10
7b	10	14±2.1	-2.3±0.2	1 - 7
	20	25±2.6	-1.3±0.3	1 - 10
8a	50	26±1.5	-1.5±0.1	1 - 10
9b	50	53±3.1	-0.9±0.09	1 - 10
5-FU	50	27±2.1	-1.1±0.1	1 - 10

A non-significant inhibition means that the treated group was not significantly different from the untreated group of mice that lost 10% lean body mass within 4-5 days and this lead to the termination of the experiment as required by the home office regulations.

The brominated pyrrol 6 was the most potent analogue of the series and its antibacterial properties were reported [20].

Derivatives of methacrylic acid[21]were identified as lead structures (scheme 2) and the synthetic analogues, derived from mucochloric acid exhibited cancer-modulating properties. The 20 mg/kg dose of derivative 7b occured a 25% tumour inhibition, comparable with the 5-FU standard, but the lower dose (10mg/kg) failed to prevent weight loss associated with the MAC16 cell line (TABLE 2).

The evaluation of weight is a significant parameter in addition of the assessment of the tumour weight.

The determination of inhibition of tumour weight is a direct parameter in order to evaluate the anticancer

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properties of the agent. A characteristic of the solid MAC16 tumour is the induction of weight loss in mice, which is reduced by an antineoplastic agent. Acute toxicity increased the weight loss above the level, which is caused by the MAC 16 tumour.

The amido-furanones 8a-c were found *in vitro* active in the micromolar range and for 8a a selectivity towards the MAC 16 cell line was determined *in vitro* (TABLE 1). The antineoplastic effect of the 50mg/kg dose is similar *in vivo* to the 5-FU standard and justifies further investigations and development.

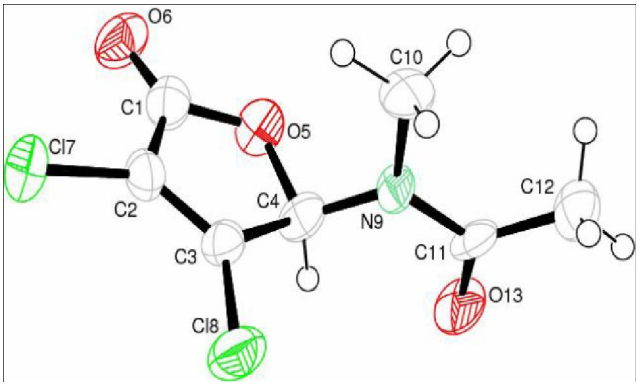

Figure 3 : Crystal structure of N-(3,4-Dichloro-5-oxo-2,5-dihydro-furan-2-yl)-N-methyl-acetamide 8a

The acetamido-furanone analogue 8a is the methylated solid derivative of the original lead structure with a molecular weight of 210. The typical dual acting effect of the test agent on tumour growths inhibition and inhibition of MAC16 induced weight loss was here clearly observed for the optimized acetamide derivative 8a (TABLE 2).

The original amido furanone lead structure 8b was prepared by second alternative synthesis and the improved derivative 8a was analysed additionally by x-ray crystallography (Figure 3).

The pyridazone 9 (Figure 4) differs in one nitrogen atom from the original pyridindione lead structure (Figure 2), but the unsaturated di-chloro-moiety was maintained. Only the unsubstituted pyridazine (R=H) analogue 9b showed a remarkable *in vitro* inhibition, which was found 7 times higher than the arylated analogue 9a for both the MAC13 and the MAC16 cell line (TABLE 1).

In vivo in mice more than 50 % inhibition of tumor growth was found for the 50mg/kg dose and this inhibition of tumour growths correlated well with the inhibition of MAC16 induced weight loss (TABLE 2).

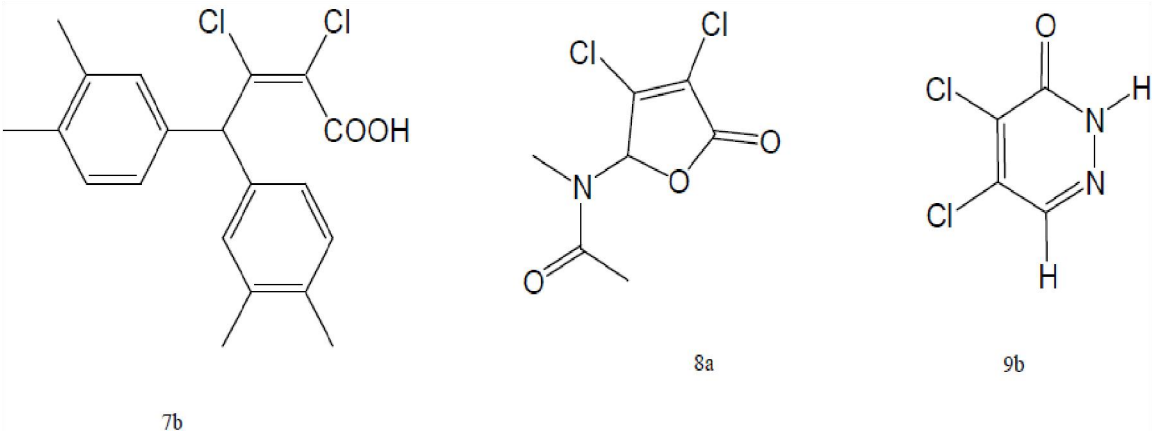

Figure 4 : Overview of optimised antineoplastic agents

This strong antineoplastic action may be due to a dual acting mechanism, in which the derivative may act as antimetabolite with alkylating properties. 9b contains an unsaturated system with chemical reactive chlorine that is prone to nucleophilic substitution (alkylating agent).

Route of administration: IP in propylene glycol

The selected active most promising molecules are outlined in figure 4.

The pyridazone 9b displayed an inhibition of 52% of tumour growth at a dose of 50 mg/kg in mice when administered intraperitoneally (TABLE 2) and additionally cancer induced weight loss is prevented [22].

CONCLUSIONS

A mechanism for the formation of 3 novel lead structures (see scheme 2) was proposed and subsequently these lead structures were synthesized by alternative

chemical routes (Scheme 3 and scheme 4). The cytotoxicity of a series of molecules derived from mucochloric acid was determined *in vitro* and selected canditates were evaluated further *in vivo* in mice on the resistant MAC 16 cancer cell line.

The acetamido furanone 8a displayed a similar activity as the standard 5-FU.

The pyridazone 9b showed twice the tumour inhibition *in vivo* in just 10 days of treatment and no signs of acute toxicity were observed.

The molecule may act as an agent with at least dual action, an antimetabolite and possibly alkylating agent.

Using semicarbazide, it can be prepared on a large scale to GMP standard and a full preclinical evaluation is justified.

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