Identification of a novel anticancer compound through screening of a drug library on multicellular spheroids

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Background and objectives
A multicellular cancer spheroid model has proven to mimic the in-vivo tumors more closely compared with the conventionally used monolayer model. Thus, the spheroid model estimates the in-vivo activity more accurately than its counterpart the monolayer model. Accordingly, a library of 320 chemically diverse compounds was screened for their cytotoxicity against MCF7 human breast carcinoma spheroids, aiming for identification of novel compounds active against this type of solid tumor.
Materials and methods
MCF7 spheroids were generated in 96-well plates by a centrifugation method. The spheroids took 5 days to reach ~500-μm diameter and were ready for treatment. The initial screen was performed at 50 μM in triplicates. A dose–response study followed the initial screen. A counterscreen was carried out using RPE1 normal cell spheroids to identify the selectivity of active compounds. The acid phosphatase method was applied to measure the cytotoxicity of compounds. A clonogenic assay was used to investigate the viability of remaining cells after treatment with test compounds.
Results and conclusion
The compound (4,5-dibromo-6-oxo-1(6H)-pyridazinyl)methyl 3-chlorophenylcarbamate was identified in this study for the first time with reasonable toxicity on MCF7 cancer spheroids. This compound is suggested as a lead compound for the development of more active derivatives against solid tumors. Additionally, the multicellular spheroid model was proved as a useful and applicable platform for identification of novel compounds for the treatment of solid tumors.

Keywords:
breast carcinoma, cancer, MCF7, RPE1, screening and anticancer drug discovery, spheroids, therapeutic window

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Introduction

Despite the enormous development in understanding the molecular basis of malignant tumors, the cure rates of cancers that require systemic treatment are still limited to 4% [1]. Thus, there is a pressing need for the development for more efficient drugs for curing cancer. Cancer chemotherapy development started since the mid-20th century. The most organized large system for drug discovery of anticancer drugs is the Developmental Therapeutic Program run by the National Cancer Institute. Paclitaxel is a prominent example of approved antineoplastic agents that have been discovered by Developmental Therapeutic Program [1]. Most currently used chemotherapeutic agents were identified in cell-based cytotoxicity assays where cancer cells are grown as monolayers [2]. It is thought that this type of screen will continue to play an important role in cancer drug discovery. However, it was found that monolayer screening is not necessarily predictive for in-vivo activity [3]. As an attempt to model solid tumors *in vitro*, Sutherland *et al.* [4] developed the multicellular spheroid model. Spheroids were shown to be superior to monolayers in modeling solid tumors in terms of growth kinetics [5], gene expression [6], three-dimensional structure, multicellular resistance [7], and similarity of extracellular matrix [8]. Thus, spheroids are considered more efficient in predicting in-vivo anticancer activity compared with their monolayers counterpart. Indeed, there is an international increasing interest in cancer spheroid model reflected in the substantial increase in the number of cancer spheroid-related publications per year, as shown by searching PubMed website for the key words 'cancer' and 'spheroids' (Fig. 1).

In the current study, 320 chemically diverse compounds were purchased and were screened for their cytotoxic activity on MCF7 human breast carcinoma spheroids. The screen led to the identification of (4,5-dibromo-6-oxo-1(6H)-

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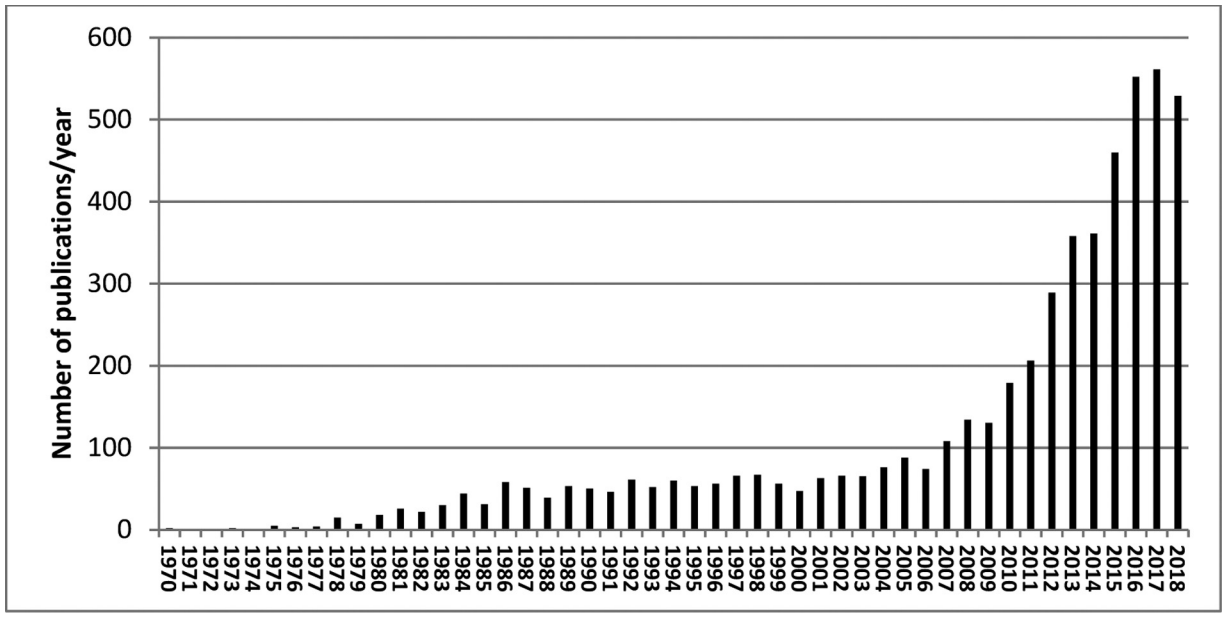
Figure 1



Number of publications per year according to searching the PubMed website for the keywords: 'cancer' and 'spheroids.'

pyridazinyl)methyl 3-chlorophenylcarbamate compound. Further dose–response and clonogenic studies on both cancer and normal spheroids showed that this compound was selectively cytotoxic toward breast cancer spheroids at concentration of 6.25 μM.

Materials and methods

Drug library

A total of 320 chemically diverse identified compounds were purchased from Specs Company (Bleiswijkseweg 552712 PB Zoetermeer, The Netherlands). The compounds were delivered in a 96-well format as dry films of 2-μmol quantities each and were dissolved in DMSO.

Cell culture

MCF7 (human breast carcinoma) and RPE1 (human normal immortalized retinal epithelial) cell lines were kindly gifted by Prof. Stig Linder, Karolinska Institute, Stockholm, Sweden. Both cell lines were grown in DMEM-F12 medium supplemented with 10% FBS and antibiotic-antimycotic (1%), and were kept at 37°C in 5% CO_2 and 95% humidity.

Generation of spheroids

Round-bottom 96-well plates coated with poly-HEMA (cat. no. P3932; Sigma, Munich, Germany) [9] were used for production of spheroids as previously described. The spheroid generation was based on the centrifugation method described by Ivascu and Kubbies [10]. Cell suspensions of 10 000 cell/well for MCF7 and 50 000 cell/well for RPE1 were seeded and centrifuged at 1000*g* for 10 min at 4°C. Plates were kept in an incubator overnight and then shaken with TITRAMAX 1000 shaker (Fisher Scientific Company, Waltham, Massachusetts, USA) at 450 rpm for further 4 days in the CO_2 incubator.

Drug treatment

Spheroids were treated for 3 h, then media was changed, and then they were incubated for further 5 days till cytotoxicity assessment. Media was changed every other day during the incubation. The initial screen was performed at 50 μM on MCF7 spheroids in triplicates. The dose–response study for the identified compound was performed on MCF7 and RPE1 spheroids at final concentrations of 50, 25, 12.5, 6.25, 3.12, and 1.56 μM in triplicates. For the study, 2-μM staurosporine was used as positive control, and 0.5% DMSO as negative control.

Cytotoxicity assessment

The acid phosphatase assay was used to measure the cytotoxicity according to the method previously described [11]. Spheroids were washed twice with 200 μl PBS, and then 100 μl of para-nitro phenyl phosphate (Santa Cruz, Heidelberg, Germany) dissolved in a buffer solution (0.1 M sodium acetate, 0.1% triton X-100, pH=5) at a concentration of 2 mg/ml was added per well and incubated for 2 h at the CO_2 incubator.

Absorbance was measured at 405 nm. Blank was subtracted from the readings, and percent cytotoxicity

was calculated by the formula [1−(D/S)] ×100, where D and S denote the optical density of drug-treated and solvent-treated spheroids, respectively.

Clonogenic assay

Both MCF7 and RPE1 spheroids were cultured and treated at the same settings performed at screening and dose–response studies. After the 5 days of incubation, the spheroids were washed with 200 µl PBS once, trypsinized for 15 min, and gently pipetted, and then spheroids were transferred to 6-well plates. The plates were incubated for further 13 days, with media change every third day. Colonies were washed with PBS, fixed with 100% methanol, stained with Geimsa and counted. The 2-µM staurosporine-treated spheroids were used as positive control, whereas 0.5% DMSO-treated spheroids were the negative control. All treatments were performed in triplicates. Clonogenicity percent reduction was calculated compared with the negative control: $[1-(N_D/N_{NC})]\times100$, where N_D is the number of colonies in the drug-treated spheroids, and N_{NC} is the number of colonies in the negative control spheroids.

Results

Screening

The drug library was screened at 50-µM final concentration on MCF7 spheroids in triplicates. Staurosporine (2 µM) was used as a positive control and caused 74.9±3.6% cytotoxicity. The % cytotoxicity results are presented in Table 1.

Dose–response studies

The compound number 237 in Table 1 was selected for further studies, as it caused the highest cytotoxicity (81%). For simplicity, the compound was given the code SP1 (Fig. 2).

A parallel dose–response study for SP1 was performed on both MCF7 and RPE1 spheroids (Fig. 3) at six concentrations: 50, 25, 12.5, 6.25, 3.12, and 1.56 µM. The results are presented in Fig. 4.

Staurosporine (2 µM) was used as a positive control and caused 76.7±2.9% cytotoxicity in MCF7 spheroids, and 81.2±4.1% cytotoxicity in RPE1 spheroids. The IC50 values of SP1 on MCF7 and RPE1 spheroids were 29 and 18 µM, respectively, computed by GraphPad Prism program.

Clonogenic assay

Both MCF7 and RPE1 spheroids were treated in triplicates at four different concentrations of SP1: 50, 25, 12.5, and 6.25 µM. The percent reduction in clonogenicity was calculated in reference to negative control (0.5% DMSO). The results are presented in Fig. 5. Staurosporine (2 µM) was used as positive control and caused 100% reduction in clonogenicity in both cell lines.

Discussion

The multicellular cancer spheroid model is gaining an increasingly interest to be employed in the anticancer drug discovery procedures. The inability of the conventional cancer monolayer model to accurately predict the in-vivo activity suits the three-dimesional models, including the cancer spheroids, in replacing the monolayer model or bridging the gap between the monolayer and animal models [12]. Of particular interest, the hypoxic quiescent subpopulation that has been found both in solid tumors and cancer spheroids represents an obstacle for achieving a successful treatment [13]. The hypoxic cells have been shown to be resistant to chemotherapeutic agent and are able to repopulate the tumor between doses [14]. This subpopulation lies far from blood vessels that carry the chemotherapy. Thus, the drug must possess a penetrating property to access these cells in a therapeutic concentration [15]. The three-dimensional cancer models are able to select for such compounds, whereas monolayers are blind to identify compounds with penetrating property.

In the present study, the breast carcinoma spheroids have been the primary platform for screening 320 chemically diverse compounds for anticancer activity. In the design of the experiment, the cancer spheroids were treated for 3 h and then media was changed aiming to mimic the physiologic conditions of clearance of the drugs from the body in comparable periods. In addition, such setting allows selection of penetrating compounds and not for cytotoxic compound that kill the spheroids layer by layer on incubating for long nonphysiological periods (>24 h). Moreover, 5 days of incubation was selected not to miss relatively late-acting drugs.

This screen identified only one compound that caused more than 80% cytotoxicity. This compound was (4,5-dibromo-6-oxo-1(6H)-pyridazinyl)methyl 3-chlorophenylcarbamate, and was termed SP1 (Fig. 2). On reviewing literature, no reported biological activity was found for this compound, and to the author's knowledge, this is the first report

Table 1 Percent cytotoxicity results of the 320 compounds on MCF7 spheroids

Number	Compound name	% CT
1	N-(4,4-dimethyl-4,5-dihydro-1H-[1,2]dithiolo[3,4-c]quinolin-1-ylidene)-N-(6-methyl-2-pyridinyl)amine	0±2.1
2	1-(7-amino-5-methyl[1,2,5]oxadiazolo[3,4-b]pyridin-6-yl)ethanone	8±3.9
3	2-(ethylsulfanyl)-6-methyl-5-(2-methyl-2-propenyl)-4-pyrimidinol	0±1.5
4	1-{[4-(4-morpholinylsulfonyl)-1-piperazinyl]carbonyl}azepane	0±2.6
5	Methyl 1H-[1]benzofuro[3,2-b]pyrrole-2-carboxylate	15 ±4.1
6	N-[2-(1-pyrrolidinyl)ethyl]benzamide	0±2.9
7	1-(4-chlorophenyl)-N-mesityl-1H-1,2,4-triazole-3-carboxamide	0±3.2
8	Methyl 4-({[4-ethyl-5-(4-methoxyphenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}methyl)benzoate	0±1.7
9	[6-(acetyloxy)-4-(anilinocarbonyl)-2-cyclohexyl-3-oxo-2,3,3a,4,5,7a-hexahydro-1H-isoindol-5-yl]methyl acetate	0±2.6
10	1-(4-chlorophenyl)-2-{[4-methyl-5-(3,4,5-trimethoxyphenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}ethanone	8±1.1
11	3-alloxycarbonyl-6-amino-5-cyano-1′,3′-dihydro-2,5′-dimethylspiro[4H-pyran-4,3′-(2′H)-indol-2′-one]	0±0.9
12	1-(4-chlorophenyl)-N-(4-isopropylphenyl)-1H-1,2,4-triazole-3-carboxamide	1±2.3
13	1-(4-chlorophenyl)-N-(3,5-dimethylphenyl)-1H-1,2,4-triazole-3-carboxamide	1±0.4
14	2-(2-methylpiperidin-1-yl)-2-oxoethyl thiocyanate	0±0.3
15	1-({2-[(2-oxopropyl)sulfanyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4-yl}sulfanyl)acetone	0±0.4
16	1,1,3,3,7,7,9,9-octamethyl-5,10,11-trithiadispiro[3.1.3.2]undecane-2,8-dione	12 ±2.7
17	Methyl 4-({[3-(trifluoromethyl)-2-pyridinyl]sulfanyl}methyl)benzoate	23 ±3.1
18	2-(2,5-dimethyl-1H-pyrrol-1-yl)-4-phenyl-1,3-thiazole	2±0.9
19	4-{4-nitro-3-methyl-5-isoxazolyl}-1-(4-methoxyphenyl)-3-phenyl-1-butanone	9±1.2
20	ethyl 4-{[2-hydroxy-5-(4-morpholinylsulfonyl)benzoyl]amino}benzoate	0±2.8
21	2-amino-4-{3-(ethyloxy)-4-[(1-methylethyl)oxy]phenyl}-7-methyl-5-oxo-4H,5H-pyrano[4,3-b]pyran-3-carbonitrile	12 ±1.9
22	1-(4-chlorophenyl)-N-methyl-N-(4-methylphenyl)-1H-1,2,4-triazole-3-carboxamide	14 ±3.1
23	9-chloro-4-(4-methylphenyl)-5,6-dihydro-4H-pyrrolo[1,2-a][1,4]benzodiazepine	21 ±2.2
24	N-(3,5-difluorophenyl)-4-(1H-pyrazol-1-yl)benzamide	13 ±1.9
25	N-(3-cyano-4,5,6,7,8,9-hexahydrocycloocta[b]thiophen-2-yl)tetrahydro-2-furancarboxamide	13 ±2.4
26	4-(1H-pyrazol-1-yl)-N-[4-(trifluoromethyl)phenyl]benzamide	0±0.3
27	1-{[4-(mesitylsulfonyl)-1-piperazinyl]methyl}-1H-benzimidazole	1±0.1
28	4-chloro-N-[2-(hydroxymethyl)phenyl]benzenesulfonamide	0±1
29	N-allyl-1-butyl-2-imino-10-methyl-5-oxo-1,5-dihydro-2H-dipyrido[1,2-a:2,3-d]pyrimidine-3-carboxamide	0±3.2
30	N-(2-methylphenyl)-3-phenyl-3-[(trifluoroacetyl)amino]propanamide	0±2.1
31	N-[1-(2-chloro-6-fluorobenzyl)-3,5-dimethyl-1H-pyrazol-4-yl]-2,2,2-trifluoroacetamide	0±0.4
32	3,5-dimethoxy-N-{4-[(4-methyl-1-piperidinyl)sulfonyl]phenyl}benzamide	19 ±1.3
33	N-{2-[(allylamino)carbonyl]phenyl}-2-furamide	29 ±4.8
34	methyl 3-[(3-methoxybenzoyl)amino]-4-(4-methyl-1-piperazinyl)benzoate	18 ±2.8
35	N-(2-benzoyl-4-bromophenyl)-4-(1-piperidinylcarbonyl)-1H-imidazole-5-carboxamide	38 ±3.2
36	N-{4-[4-(2-furoyl)-1-piperazinyl]phenyl}-N'-(2-naphthoyl)thiourea	7±1.7
37	5-bromo-N-[5-methoxy-2-(4-morpholinyl)phenyl]-2-furamide	16 ±2.3
38	1-(4-chlorophenyl)-N-[3-(difluoromethoxy)phenyl]-1H-1,2,4-triazole-3-carboxamide	0±1
39	methyl 3-phenyl-1-isoquinolinyl sulfide	0±3.5
40	1-isopropoxy-3-[(1,1,3,3-tetramethylbutyl)amino]-2-propanol	0±1.4
41	ethyl 7-oxo-5-phenyl-2,3,3a,6,7,7a-hexahydrofuro[2,3-c]pyridine-4-carboxylate	0±3.9
42	N-[4-(aminosulfonyl)benzyl]-3-(1H-1,2,4-triazol-1-yl)-1-adamantanecarboxamide	0±2.5
43	N-{4-[(3,4-dichlorobenzoyl)amino]-2-methoxyphenyl}-2-furamide	29 ±4.7
44	ethyl 6-ethoxy-3-hydroxy-2-oxo-7-phenyl-2,5-dihydro-1H-azepine-4-carboxylate	4±1.2
45	N-cyclopentyl-1-[[(2,4-dichlorophenoxy)acetyl](methyl)amino]cyclohexanecarboxamide	33 ±2.9

(*Continued*)

Table1 (*Continued*)

Number	Compound name	% CT
46	2-[benzyl(methylsulfonyl)amino]-N-[2-(trifluoromethyl)phenyl]acetamide	0±2
47	3-(3-bromo-1H-1,2,4-triazol-1-yl)-N-(3-chloro-4-fluorophenyl)-1-adamantanecarboxamide	36 ±3.4
48	2-[[(4-bromophenyl)sulfonyl](methyl)amino]-N-(2,6-difluorophenyl)acetamide	0±2.6
49	3-(3,4-dimethoxyphenyl)-5-[(4-methoxybenzyl)sulfanyl]-4-phenyl-4H-1,2,4-triazole	9±1.2
50	2-ethyl-N-(5-ethyl-1,3,4-thiadiazol-2-yl)hexanamide	6±0.8
51	2-amino-N-(2-methoxyphenyl)-6-methyl-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxamide	0±3.3
52	3-amino-2-[(2-oxo-2-phenylethyl)sulfanyl]-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one	4±1.1
53	N-[5-(1H-benzimidazol-2-yl)-2-methylphenyl]-2-(2-chlorophenoxy)acetamide	10 ±2.8
54	4-(acetylamino)phenyl dimethylsulfamate	0±1.5
55	2-[5-cyclopropyl-3-(trifluoromethyl)-1H-pyrazol-1-yl]-N-(4-pyridinylmethyl)acetamide	0±3.1
56	N-[4-(isobutyrylamino)-3-methoxyphenyl]-2-thiophenecarboxamide	0±2.8
57	4-nitro-1-methyl-N-[4-(trifluoromethoxy)phenyl]-1H-pyrazole-3-carboxamide	26 ±4.7
58	N-[4-({6-[(3-pyridinylmethyl)amino]-4-pyrimidinyl}sulfanyl)phenyl]acetamide	0±3.6
59	[6,8-dimethyl-1,2-bis(propylsulfonyl)-3-indolizinyl](4-fluorophenyl)methanone	0±2.1
60	N-(6-chloro-2-pyridinyl)-4-ethoxybenzamide	0±2.6
61	2-propionyl-3,3-bis(trifluoromethyl)-2-azabicyclo[2.2.1]hept-5-ene	14 ±1.2
62	3,4-dimethoxy-N-(2-phenoxyethyl)benzamide	17±5
63	8-(4-morpholinylmethyl)-3-(2-pyrazinyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-a]pyrimidin-6-ol	6±0.7
64	N-(6-acetyl-1,3-benzodioxol-5-yl)-2,4-dibromobenzamide	4±0.5
65	1-(2,3-dihydro-1H-inden-5-yloxy)-3-(3,4,5-trimethyl-1H-pyrazol-1-yl)-2-propanol	11±1
66	2-{[5-(3-fluorophenyl)-4-phenyl-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(2-phenoxyethyl)acetamide	9±2.1
67	1-(2-methylbenzoyl)-N-[4-(trifluoromethyl)phenyl]-4-piperidinecarboxamide	12 ±4.4
68	N-(4-fluorophenyl)-12,15,15-trimethyl-3,10-diazatetracyclo[10.2.1.0∼2,11∼.0∼4,9∼]pentadeca-2(11),3,5,7,9-pentaene-1-carboxamide	19 ±2.6
69	N-cyclopentyl-1,2,3-trimethyl-1H-indole-5-carboxamide	0±0.3
70	2-(1H-benzimidazol-2-ylsulfanyl)-N-[4-(pentyloxy)phenyl]acetamide	0±0.5
71	2-{[5-(3,4-dimethoxyphenyl)-4-phenyl-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(4-ethylphenyl)acetamide	5±1.1
72	(5-benzyl-4H-1,2,4-triazol-3-yl)acetonitrile	16 ±2.4
73	2-phenoxybicyclo[6.2.0]deca-2,4,6-triene-9,9,10,10-tetracarbonitrile	10 ±1.7
74	dimethyl-4-phenyl-1H-pyrazole-3,5-dicarboxylate	3±0.4
75	2′-(anilinocarbonyl)[1,1′-biphenyl]-2-carboxylic acid	6±0.4
76	2-(3-cyclohexylpropyl)-1-(methylsulfonyl)-1H-benzimidazole	0±1.1
77	2-(3,4-dimethoxybenzyl)-1H-benzimidazole	4±0.8
78	N-(4-{2-hydroxy-3-[4-(mesitylsulfonyl)-1-piperazinyl]propoxy}phenyl)acetamide	22 ±3.6
79	5-(4-ethylphenyl)-3-hydroxy-1-[2-(2-hydroxyethoxy)ethyl]-4-(2-thienylcarbonyl)-1,5-dihydro-2H-pyrrol-2-one	4±1.9
80	ethyl 1-(4-methylphenyl)-3-phenyl-1H-pyrazole-4-carboxylate	0±0.4
81	N-(2-chloro-6-fluorobenzoyl)-N'-(2-naphthyl)urea	0±0.7
82	6-amino-5-benzyl-5H-[1,2,5]oxadiazolo[3,4-b]pyrrolo[2,3-e]pyrazine-7-carbonitrile	0±1.4
83	3-{4-ethoxy[(methylamino)carbonyl]anilino}-2-methylpropanoic acid	0±1.9
84	3-[2-(4-chlorophenyl)-5-oxo-5,6-dihydro-4H-1,3,4-oxadiazin-4-yl]propanenitrile	0±4.5
85	ethyl 2-[(2-bromobenzoyl)amino]-5-[(diethylamino)carbonyl]-4-methyl-3-thiophenecarboxylate	3±2.1
86	1-(4-chlorophenyl)-4-(1H-naphtho[2,3-d]imidazol-2-yl)-2-pyrrolidinone	5±1.2
87	2-amino-4-(2-bromo-4-fluorophenyl)-5-oxo-±4H,5H-pyrano[3,2-c]chromene-3-carbonitrile	0±2.9
88	3,4,5-trimethoxy-N-[2-(1-pyrrolidinyl)-5-(trifluoromethyl)phenyl]benzamide	6±3.2
89	6-amino-8-(4-isopropoxy-3-methoxyphenyl)-2-methyl-2,3,8,8a-tetrahydro-5,7,7(1H)-isoquinolinetricarbonitrile	0±2.6
90	3-phenyl-4-thia-1,2-diazaspiro[4.7]dodec-2-ene	10 ±2.7
91	N-(2-iodobenzyl)benzenecarbothioamide	0±0.8
92	N-(4-bromo-2,3,5,6-tetrafluorophenyl)-2-{4-nitro-3,5-dimethyl-1H-pyrazol-1-yl}acetamide	0±2.8
93	ethyl 4-[2-(2,4-difluorophenyl)-2-hydroxy-3-(1H-1,2,4-triazol-1-yl)propoxy]benzoate	0±3.1

(*Continued*)

Table1 (*Continued*)

Number	Compound name	% CT
94	(3-methyl-1-isoquinolinyl)(phenyl)methanone	10 ±0.5
95	N-[4-(3-ethyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)phenyl]-2,2-dimethylpropanamide	11 ±1.3
96	2-(4-morpholinyl)-2-oxoethyl thiocyanate	0±0.9
97	6-methyl-5H-[1,2,4]triazolo[3′,4′:2,3][1,3]thiazino[5,4-c]quinolone	0±0.4
98	N-benzyl-2-methyl-3-furamide	14±
99	1-(4-butoxy-3-{[4,6-dihydroxy-2-(methylsulfanyl)-5-pyrimidinyl]methyl}phenyl)ethanone	15 ±1.5
100	2-acetyl-3-(2-furyl)-7-(2-furylmethylene)-3,3a,4,5,6,7-hexahydro-2H-indazole	14 ±3.9
101	4-ethoxy-N-[4-(3-ethyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl)phenyl]benzamide	0±5.8
102	methyl 3-(trifluoroacetyl)-1,3-thiazolidine-4-carboxylate 1,1-dioxide	0±2.7
103	2-{[5-(phenoxymethyl)-1,3,4-oxadiazol-2-yl]sulfanyl}-1-phenylethanone	5±0.1
104	N-{3-chloro-4-[4-(2-furoyl)-1-piperazinyl]phenyl}-4-methoxybenzamide	17 ±1.2
105	2-[2-(dimethylamino)benzylidene]-1-benzofuran-3(2H)-one	20 ±4.6
106	N-(2-{5-[2-hydroxy-3-(isopropylamino)propoxy]-1H-indol-3-yl}ethyl)acetamide	3±0.5
107	3-{1-[2-hydroxy-3-(4-morpholinyl)propyl]-1H-indol-3-yl}-1-(4-methoxyphenyl)-2-propen-1-one	7±1.3
108	2-(2-hydroxy-3-methoxybenzylidene)-5-methoxy-1-benzofuran-3(2H)-one	27 ±1.8
109	1-[1-(3-chlorophenyl)-4-(2-thienylcarbonyl)-1H-pyrazol-3-yl]ethanone	0±3.5
110	4-cyanophenyl 4-(butylamino)benzoate	6±1.1
111	butyl (6-bromo-4-oxo-4H-chromen-3-yl)carbonyl carbamate	0±2
112	1-[(3-methyl-1-phenyl-1H-1,2,4-triazol-5-yl)methyl]-2-pyrrolidinylidenecyanamide	0±4.1
113	4-ethoxy-N-[4-(isobutyrylamino)-3-methoxyphenyl]benzamide	5±1.2
114	N-(3-acetylphenyl)-N'-(3-chlorobenzoyl)thiourea	11 ±1.6
115	N∼2∼-butyl-N∼3∼-(3-fluorophenyl)-5,6-dimethyl-2,3-pyrazinedicarboxamide	9±1.4
116	ethyl 5-{[(4-chloro-3-methylphenoxy)acetyl]amino}-4-cyano-3-methyl-2-thiophenecarboxylate	7±0.4
117	dimethyl 10-amino-6-oxo-6H-benzo[h]thiepino[3,2-c]chromene-8,9-dicarboxylate	53 ±6.4
118	N∼2∼-butyl-N∼3∼-(2-chlorophenyl)-5,6-dimethyl-2,3-pyrazinedicarboxamide	0±2.9
119	3-[(3-bromo-4-methoxybenzyl)sulfanyl]-5-methyl-4H-1,2,4-triazole	11 ±1.3
120	N∼2∼-(4-chlorophenyl)-5,6-dimethyl-N∼3∼-propyl-2,3-pyrazinedicarboxamide	16 ±2.3
121	5-amino-12-methyl-3-(3-thienyl)-12-azatricyclo[7.2.1.0∼2,7∼]dodeca-5,7-diene-4,4,6-tricarbonitrile	0±3.1
122	N-(3-chlorobenzoyl)-N'-[2-(hydroxymethyl)phenyl]thiourea	0±0.2
123	N-[2-(2-oxo-1-pyrrolidinyl)ethyl]-N'-phenylthiourea	13 ±1.6
124	benzo[g]phthalazine-1,4-diol	18 ±2.7
125	1-[2-(4-morpholinyl)-2-thioxoethyl]-2-pyrrolidinethione	3±0.5
126	dimethyl-2-(2,2-dimethyl-3-thioxo-2,3-dihydro-4(1H)-quinolinylidene)-3-(3-methylphenyl)-2,3-dihydro-1,3-thiazole-4,5-dicarboxylate	32 ±4.5
127	ethyl 3-(anilinocarbonyl)-2-pyrazinecarboxylate	0±1.1
128	N-{2-chloro-4-[(4-isopropoxybenzoyl)amino]phenyl}-2-furamide	23 ±4.3
129	tert-butyl 2-amino-5′-bromo-2′,5-dioxo-1′,3′,5,6,7,8-hexahydrospiro[4H-chromene-4,3′-(2′H)-indole]-3-carboxylate	18 ±1.3
130	5-methoxy-2-[3-(2-methoxyphenyl)acryloyl]phenyl acetate	34 ±4.1
131	propyl 3-(anilinocarbonyl)-2-pyrazinecarboxylate	0±0.7
132	2-(allylsulfanyl)-5,6-dihydrospiro(benzo[h]quinazoline-5,1′-cyclopentane)-4(3H)-one	0±1
133	di(tert-butyl) 8-oxo-1,3,4-triazabicyclo[4.2.0]oct-2-ene-2,4-dicarboxylate	9±2.1
134	2-benzyl-N-(2,2-dimethyl-4-oxo-3-thietanyl)-1,3-oxazole-4-carboxamide	12±
135	7,8,9,10-tetrahydrobenzo[def]chrysene-7,8-diol	10 ±3.8

(*Continued*)

Table1 (***Continued***)

Number	Compound name	% CT
136	2,5-dichloro-N-{4-[4-(methylsulfonyl)-1-piperazinyl]phenyl}benzamide	4±0.6
137	3-({[5-(ethoxycarbonyl)-4-methyl-1,3-thiazol-2-yl]amino}carbonyl)bicyclo[2.2.1]hept-5-ene-2-carboxylic acid	10 ±1.4
138	4-[(2,2-dibromo-1-methylcyclopropyl)carbonyl]morpholine	3±0.8
139	propyl 3-({4-[(4-chlorobenzyl)oxy]anilino}carbonyl)-2-pyrazinecarboxylate	15 ±1.9
140	N-[3-({[(phenylacetyl)amino]carbothioyl}amino)phenyl]propanamide	7±0.3
141	N-[2-(difluoromethoxy)phenyl]-3,4-dimethylbenzamide	0±0.4
142	methyl 2-(benzyloxy)-4-methylbenzoate	0±1.9
143	ethyl 3-[(3,5-dichloroanilino)carbonyl]-2-pyrazinecarboxylate	14 ±1.8
144	N-{4-chloro-3-[(2,2-dimethylpropanoyl)amino]phenyl}butanamide	0±0.7
145	N-{3-[(3-methoxybenzoyl)amino]phenyl}-2-thiophenecarboxamide	25 ±3.8
146	1-[(2,5-dichlorophenyl)sulfonyl]-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile	6±0.7
147	4-amino-5-methoxy-9(10H)-acridinone	9±2.7
148	6-(2-hydroxyphenyl)-4-(4-isopropylphenyl)-2-oxo-1,2-dihydro-3-pyridinecarbonitrile	24 ±3.6
149	2-(2-acetyl-1H-benzimidazol-1-yl)-1-(4-methylphenyl)ethanone	22 ±2.9
150	2-{4-bromo-3-nitro-5-methyl-1H-pyrazol-1-yl}-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)acetamide	0±0.8
151	2-chloro-N-methyl-N-[(6-phenyl-7H-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-3-yl)methyl]benzenesulfonamide	0±1.1
152	3,3,6,6-tetramethyl-9-phenyl-3,4,5,6,7,9-hexahydro-1H-xanthene-1,8(2H)-dione	8±5.8
153	2-oxo-2-phenylethyl 2,4-dichloro-5-[(diethylamino)sulfonyl]benzoate	10 ±4.1
154	N-allyl-N'-(1H-1,2,3-benzotriazol-1-ylmethyl)thiourea	0±3.3
155	3-(6-chloro-3-oxo-2,3-dihydro-4H-1,4-benzoxazin-4-yl)propanenitrile	12 ±1.3
156	2-methylquinazolino[2,3-c][1,4]benzoxazin-12(6H)-one	5±2.1
157	N-benzyl-N-(5-bromo-2-pyridinyl)amine	5±1.7
158	2-[([1,1′-biphenyl]-4-yloxy)methyl]-8-methylimidazo[1,2-a]pyridine	25 ±5.2
159	N-(dicyclopentylmethyl)benzamide	17 ±3.9
160	methyl 4-cyano-5-(2-furoylamino)-3-methyl-2-thiophenecarboxylate	22 ±2.5
161	N-(5-methyl-1,3,4-thiadiazol-2-yl)-4-(4-morpholinylsulfonyl)benzamide	0±1.8
162	2-(4-iodo-3,5-dimethyl-1H-pyrazol-1-yl)succinic acid	0±0.6
163	N-[(5-{[2-(4-bromophenyl)-2-oxoethyl]sulfanyl}-4-methyl-4H-1,2,4-triazol-3-yl)methyl]-3-methylbenzamide	0±0.4
164	ethyl 4-(1,3-benzothiazol-2-ylcarbonyl)-1-(4-methoxyphenyl)-1H-pyrazole-3-carboxylate	0±2.1
165	2-{[4-ethyl-5-(phenoxymethyl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(3-methoxyphenyl)acetamide	0±3
166	N-[(1-phenylcyclopentyl)methyl]acetamide	0±1.3
167	1,2-diacetyl-3,6-diphenyl-1,2-dihydro-1,2,4,5-tetraazine	0±0.4
168	methyl 3-(2,4,6-trimethoxyphenyl)-5-isoxazolecarboxylate	0±0.5
169	2-amino-N-(4-methylphenyl)-4-oxo-5,6-dihydro-4H-1,3-thiazine-6-carboxamide	0±2.2
170	3-isopropoxy-N-[5-(4-methoxybenzyl)-1,3,4-thiadiazol-2-yl]benzamide	10 ±0.7
171	2-[2-methoxy-5-methyl(methylsulfonyl)anilino]-N-(3-pyridinyl)acetamide	3±0.4
172	1-[(2-bromo-4-isopropylphenoxy)acetyl]-4-(2-methoxyphenyl)piperazine	6±2.2
173	3-({[5-(2-chlorophenyl)-4H-1,2,4-triazol-3-yl]sulfanyl}acetyl)-3,8,8-trimethyl-2,7-dioxaspiro[4.4]nonane-1,6-dione	0±2.3
174	[4-(4-morpholinyl)phenyl](2-thienyl)methanone	0±1.8
175	S-ethyl 2-pyridinylmethylthiocarbamate	0±0.7
176	7-hydroxy-6-methoxy-1-benzothiophen-2(3H)-one	0±1.4
177	N-(1,1-dimethyl-2-propynyl)-2-[4-methoxy(phenylsulfonyl)anilino]acetamide	0±1.5
178	N-cyclopropyl-2-{[(4-fluorophenyl)sulfonyl]-4-methylanilino}acetamide	0±0.9
179	N-(1-adamantyl)-2-[(1-phenyl-1H-tetraazol-5-yl)sulfanyl]acetamide	0±0.4
180	5-(4-bromophenyl)-N-phenyl-2-furamide	0±1.4
181	5,5-dimethyl-2-(2-thienyl)-1,10b-dihydropyrazolo[1,5-c][1,3]benzoxazine	7±1.1
182	4-(3,4-dimethoxyphenyl)-N-[3-(trifluoromethyl)phenyl]tetrahydro-2H-pyran-4-carboxamide	0±0.6

(*Continued*)

Table1 (*Continued*)

Number	Compound name	% CT
183	N-(3-ethoxypropyl)-2-(4-fluorophenyl)acetamide	0±1.7
184	2-[benzoyl(cyano)amino]ethyl benzoate	0±0.9
185	N-butyl-2-[[(4-methoxyphenyl)sulfonyl](methyl)amino]acetamide	0±0.5
186	4-{[4-(1,1-dioxido-1,2-benzisothiazol-3-yl)-1-piperazinyl]carbonyl}-N,N-diethylbenzenesulfonamide	0±1.3
187	2-(5-bromo-2-hydroxyphenyl)-5,7-dimethyl-1,3-diazatricyclo[3.3.1.1∼3,7∼]decan-6-ol	0±2.4
188	2-[(2,4-dimethylbenzyl)sulfanyl]-5-isopentyl-6-methyl-4(3H)-pyrimidinone	0±0.1
189	methyl 3-({4-[4-(trifluoromethyl)benzyl]-1-piperazinyl}methyl)phenyl ether	16 ±1.5
190	5-(4-methylphenyl)-7-(trifluoromethyl)-N-[(1,3,5-trimethyl-1H-pyrazol-4-yl)methyl]pyrazolo[1,5-a]pyrimidine-2-carboxamide	0±0.7
191	(4-methoxyphenyl)(4-toluidino)acetonitrile	0±1.7
192	4-cyanophenyl 4-(3-butenyloxy)benzoate	0±3
193	[1,1′-biphenyl]-4-yl tetrahydro-2H-pyran-2-yl ether	34 ±4.5
194	2-({4-nitro-2-methyl-1H-imidazol-5-yl}sulfanyl)-1-phenylethanone	0±2.6
195	4-(4-methoxyphenyl)-3-(phenylsulfonyl)-3-buten-2-one	0±2.3
196	N-(4-fluorophenyl)-N'-(2-thienylcarbonyl)thiourea	23 ±2.2
197	N-(4-methoxyphenyl)-N'-(2-thienylcarbonyl)thiourea	2±2.1
198	1-iodo-4-phenylbicyclo[2.2.2]octane	18 ±5.5
199	N-(2,4-dimethylphenyl)-1-(3,4-dimethylphenyl)-5-oxo-3-pyrrolidinecarboxamide	8±3.9
200	1,1-dioxido-4H-pyrido[4,3-e][1,2,4]thiadiazin-3-ylamine	25 ±3.7
201	methyl pyrazolo[5,1-a]isoquinoline-1-carboxylate	0±0.7
202	ethyl 4-(4-chlorobenzoyl)-1-(4-methylphenyl)-1H-pyrazole-3-carboxylate	0±3.6
203	allyl 6-amino-5-cyano-4-(3-furyl)-2-methyl-4H-pyran-3-carboxylate	9±2.1
204	N-[4-(4-methyl-1-naphthyl)-1,3-thiazol-2-yl]butanamide	33 ±2.9
205	2-{[6-isopropyl-3-(2-methylphenyl)-4-oxo-3,5,6,8-tetrahydro-4H-pyrano[4′,3′:4,5]thieno[2,3-d]pyrimidin-2-yl]sulfanyl} propanoic acid	12 ±1.5
206	5,5a,7,8-tetrahydro-4H-[1,2,5]oxadiazolo[3,4-e]indole-6,8a-diol	18 ±4.7
207	N-benzoyl-N'-(3-cyano-6-propyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl)thiourea	0±2
208	2-{2-[4-(dimethylamino)phenyl]vinyl}-3-(4-hydroxyphenyl)-4(3H)-quinazolinone	0±0.7
209	1-(3,4-dimethylphenyl)-4-(1-pyrrolidinyl)phthalazine	20 ±2.9
210	N-(3,4-dimethoxyphenyl)-5-[(2,3,4,5,6-pentafluorophenoxy)methyl]-2-furamide	0±2.6
211	3-{[5-(4-pyridinyl)-4H-1,2,4-triazol-3-yl]sulfanyl}propanoic acid	0±0.4
212	4-cyano-5-methyl-N-(4-methylphenyl)-4-phenylhexanamide	8±0.7
213	N-[3-(1,3-benzothiazol-2-yl)phenyl]-2-fluorobenzenesulfonamide	1±0.6
214	3-[(3-cyano-4-ethyl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-2-quinolinyl)sulfanyl]-N-(4-methoxyphenyl)propanamide	11 ±0.8
215	4-[(2,5-dimethyl-3-furoyl)amino]benzoic acid	0±1.5
216	ethyl [6,7-dimethoxy-3,4-dihydrospiro(isoquinoline-3,1′-cyclopentane)-1(2H)-ylidene]acetate	17±5
217	2-{[(3,4-dimethoxyphenyl)sulfonyl]-4-methylanilino}-N-(3-pyridinyl)acetamide	0±0.7
218	2-[(3-allyl-4-oxo-3,4-dihydro-2-quinazolinyl)sulfanyl]-N,N-diphenylacetamide	0±3.9
219	3-(anilinocarbonyl)bicyclo[2.2.1]heptane-2-carboxylic acid	0±1.1
220	3-[(4-carboxyanilino)carbonyl]bicyclo[2.2.1]heptane-2-carboxylic acid	0±0.6
221	4-[3-(dimethylamino)propylidene]-4,10-dihydrothieno[2,3-c][1]benzothiepine-6-carbonitrile	0±2.4
222	1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile	0±3.6
223	dimethyl 2-[4-(dimethylamino)phenyl]-4-hydroxy-4-methyl-6-oxo-1,3-cyclohexanedicarboxylate	0±1.7
224	4-(2-{2-[(3-chlorophenoxy)methyl]-1,3-dioxolan-2-yl}vinyl)hexahydro-2H-cyclopenta[b]furan-2,5-diol	0±0.9
225	8-(dimethylamino)-6-methoxy-2-phenylhexahydropyrano[3,2-d][1,3]dioxin-7-ol	0±3.3
226	5-chloro-2-phenyl-4-(1-pyrrolidinyl)-3(2H)-pyridazinone	4±0.4
227	5-[(4-bromo-3,5-dimethyl-1H-pyrazol-1-yl)methyl]-N-(3,4,5-trimethoxyphenyl)-2-furamide	13 ±2.8
228	N-(4-fluorophenyl)-2-(4-isopropylphenoxy)acetamide	0±1.6
229	1-(2-{2-[2-(1H-benzimidazol-1-yl)ethoxy]ethoxy}ethyl)-1H-benzimidazole	0±3.2

(*Continued*)

Table1 (*Continued*)

Number	Compound name	% CT
230	2-{2-[(3-hydroxypropyl)amino]-1H-benzimidazol-1-yl}-1-(2-thienyl)ethanone	0±2.7
231	4-[(acetylamino)methyl]-2-(acetyloxy)phenyl acetate	0±0.9
232	1-(1,3-benzodioxol-5-ylmethyl)isoquinoline	0±1.2
233	1-(4-isopropyl-3-methylphenoxy)-3-[4-(2-pyridinyl)-1-piperazinyl]-2-propanol	20 ±4.2
234	3-allyl-5-(2-furyl)-2-({2-[1-(2-methoxyethyl)-2,5-dimethyl-1H-pyrrol-3-yl]-2-oxoethyl}sulfanyl)thieno[2,3-d]pyrimidin-4(3H)-one	0±0.6
235	5-[bis(2-hydroxyethyl)amino]-2-(diethylamino)benzamide	0±1.4
236	3-[(5-phenyl-2-furyl)methylene]-2,4-pentanedione	33 ±3.5
237	(4,5-dibromo-6-oxo-1(6H)-pyridazinyl)methyl 3-chlorophenylcarbamate	81 ±1.7
238	3-(3-chlorophenyl)-1-{5-nitro-2-furyl}-2-(phenylsulfonyl)-2-propen-1-one	23 ±1.1
239	2-phenyl-N-(1,3-thiazol-2-yl)butanamide	0±0.4
240	2-{[1-(4-ethoxyphenyl)-1H-tetraazol-5-yl]sulfanyl}-N-isopropyl-N-phenylacetamide	0±1.6
241	methyl 6-amino-4-{5-[(4-chlorophenoxy)methyl]-2,4-dimethylphenyl}-5-cyano-2-methyl-4H-pyran-3-carboxylate	5±1.9
242	2-(2-bromo-6-methoxy-4-{[(1-phenylethyl)amino]methyl}phenoxy)-N-(tert-butyl)acetamide	13 ±0.6
243	N-(3-ethoxyphenyl)-2,3-dimethoxybenzamide	0±1.3
244	2-{4-[(2-adamantylamino)methyl]phenoxy}-N-(tert-butyl)acetamide	6±2.1
245	4-({[(diphenylacetyl)amino]carbothioyl}amino)-N-isopropylbenzenesulfonamide	9±2.6
246	(2-bromo-5-ethoxy-4-methoxyphenyl)methanol	0±0.7
247	4-[6-(4-ethylphenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl]phenyl methyl ether	0±1.1
248	4-(butoxycarbonyl)phenyl 3-methoxybenzoate	0±2
249	phenyl 2-phenylhexahydropyrano[3,2-d][1,3]dioxin-8-yl carbonate	0±0.7
250	2-[cyclohexyl(methyl sulfonyl)amino]-N-(2-phenylethyl)acetamide	0±2.7
251	7-(2,3-dimethylphenyl)-7H-[1,2,3]triazolo[4,5-e][2,1,3]benzoxadiazole	0±0.4
252	N-{amino[(6-ethyl-4-methylquinazolin-2-yl)amino]methylene}-4-methylbenzenesulfonamide	0±2.1
253	6-amino-3-(1,3-benzodioxol-5-yl)-4-(3-bromo-4-fluorophenyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile	4±0.9
254	2-(2,3-dimethyl-1H-indol-1-yl)-2-oxoethyl 4-methoxybenzoate	0±1.7
255	3-chloro-N-{4-[(4-phenyl-1-piperazinyl)carbonyl]benzyl}benzenesulfonamide	0±2.2
256	2-(4-ethoxyphenyl)-3-(6-methoxy-1,3-benzothiazol-2-yl)-1,3-thiazolidin-4-one	0±2.2
257	1-[(5-bromo-2-thienyl)sulfonyl]-4-piperidinecarboxylic acid	0±0.8
258	2-(2-methylphenoxy)-N-(2-phenylethyl)propanamide	0±3.3
259	ethyl 1-phenyl-5-[(4-toluidinocarbonyl)amino]-1H-pyrazole-4-carboxylate	0±1.8
260	2-(1,3-benzoxazol-2-ylsulfanyl)-N-(3-methyl-1-phenyl-1H-pyrazol-5-yl)acetamide	0±0.5
261	ethyl 2-[(2-methoxyanilino)methyl]-5-methyl-4-oxo-3,4-dihydrothieno[2,3-d]pyrimidine-6-carboxylate	0±2.1
262	3-(4-bromophenyl)-5-(2-furyl)-1-(tetrahydro-2-furanylcarbonyl)-4,5-dihydro-1H-pyrazole	20 ±3.7
263	1-(3-chloro-2-methylphenyl)-N-(2,3-dimethylphenyl)-5-oxo-3-pyrrolidinecarboxamide	0±0.8
264	3-(2-fluorobenzyl)-6-isopropyl[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	0±0.4
265	2-(cyclopropylamino)-2-oxoethyl N'-cyano-N-(4-ethoxyphenyl)imidothiocarbamate	0±0.7
266	1-(3,4-dimethylphenyl)-N-(4-iodophenyl)-5-oxo-3-pyrrolidinecarboxamide	0±3.2
267	N-{4-[3-(4-bromophenyl)-3-oxo-1-propenyl]phenyl}propanamide	3±1.3
268	2-(isopentylsulfanyl)-6-(2-thienyl)nicotinonitrile	15 ±0.7
269	2,4-dichloro-N-[2-(hydroxymethyl)phenyl]benzamide	2±1.4
270	methyl 5-amino-1-[3-(trifluoromethyl)phenyl]-1H-1,2,3-triazole-4-carboxylate	0±4.4
271	3-[(cyclopropylcarbonyl)amino]-N-(2-ethoxyphenyl)benzamide	0±0.8
272	4-[6-(4-fluorobenzyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl]phenyl methyl ether	0±1.8
273	methyl 4-[6-(2-phenylethyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl]phenyl ether	1±2.5
274	6-(ethoxymethyl)-3-(4-methoxyphenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	0±0.8
275	dimethyl 5-{[2-(4-chlorobenzoyl)benzoyl]amino}isophthalate	0±1.7
276	6-(4-methoxybenzyl)-3-(4-methoxyphenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	5±0.6
277	4-(2-chlorophenyl)-N,N-dimethyl-1-piperazinesulfonamide	0±3.5
278	2-{[(4-methylphenyl)sulfonyl]methyl}-5-phenylthieno[2,3-d]pyrimidin-4(3H)-one	0±0.9
279	4-(4-chlorophenyl)-8,10-dimethyl-3,4-dihydro-1H-pyrido[3′,2′:4,5]furo[3,2-e][1,4]diazepine-2,5-dione	0±4.3

(*Continued*)

Table1 (*Continued*)

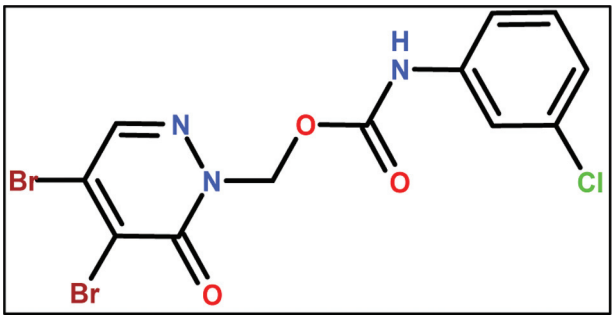
Number	Compound name	% CT
280	2-{[4-(4-bromophenyl)-1-(2-methylphenyl)-1H-imidazol-2-yl]sulfanyl}acetamide	10 ±2.3
281	4-[(dipropylamino)sulfonyl]-N-(2-furylmethyl)benzamide	0±0.1
282	2-(4-chlorobenzoyl)-N-isobutyl benzamide	0±2.1
283	4-tert-butylphenyl [3-(trifluoromethyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl]methyl ether	0±3.1
284	5-amino-1-(6-methoxy-1,3-benzothiazol-2-yl)-3-methyl-1H-pyrazole-4-carbonitrile	0±1.6
285	4-[6-(2-bromophenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl]phenyl methyl ether	10 ±1.3
286	4-[6-(2,4-dimethylphenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-3-yl]phenyl methyl ether	0±0.8
287	methyl 5-amino-1-(1,3-benzothiazol-2-yl)-3-(methylsulfanyl)-1H-pyrazole-4-carboxylate	0±1.3
288	6-(2-methoxyphenyl)-3-(4-methoxyphenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	4±0.3
289	3-[(2-oxo-2-phenylethyl)thio]-6-(3-pyridinylmethyl)-1,2,4-triazin-5(4H)-one	0±2.2
290	2-{[4-(ethylamino)-7-methyl-5,6,7,8-tetrahydropyrido[4′,3′:4,5]thieno[2,3-d]pyrimidin-2-yl]sulfanyl}acetamide	0±0.2
291	N-{3-[(3,5-dimethoxybenzoyl)amino]propyl}isonicotinamide	0±0.9
292	N-(3-acetylphenyl)-2-[1,1′-biphenyl]-4-ylacetamide	0±2.7
293	6-(methoxymethyl)-3-(4-methoxyphenyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	13 ±3.8
294	4-bromo-N-(3,5-dimethyl-1-phenyl-1H-pyrazol-4-yl)benzamide	0±1.6
295	N-[1,1′-biphenyl]-4-yl-4-[(dimethylamino)sulfonyl]benzamide	0±0.7
296	methyl 2-({4-[(dimethylamino)sulfonyl]benzoyl}amino)-3,4,5-trimethoxybenzoate	0±3.2
297	3-bromo-N-(4-{[(tetrahydro-2-furanylmethyl)amino]carbonyl}phenyl)benzamide	7±2.5
298	N-{2-[(3-butyl-1-isoquinolinyl)oxy]ethyl}-N,N-dimethylamine	1±0.4
299	3,4,5-triethoxy-N-(1-naphthyl)benzamide	0±0.7
300	dimethyl 5-[(5-chloro-2-methoxybenzoyl)amino]isophthalate	0±0.5
301	4-chlorophenyl [3-(trifluoromethyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl]methyl ether	0±1.6
302	N-(3-{[2-(4-methylphenoxy)acetyl]amino}propyl)nicotinamide	14 ±3.7
303	N-(3-{[2-(2-chlorophenoxy)acetyl]amino}propyl)nicotinamide	17 ±4.1
304	N-{2-[(2-fluorobenzoyl)amino]ethyl}-2-pyridinecarboxamide	0±0.9
305	N-{2-[(3,4-dimethoxybenzoyl)amino]ethyl}-2-pyridinecarboxamide	0±0.9
306	N-(2-{[2-(4-methoxyphenoxy)acetyl]amino}ethyl)-2-pyridinecarboxamide	0±2.4
307	N-(2-{[2-(3-methylphenoxy)acetyl]amino}ethyl)-2-pyridinecarboxamide	0±4.3
308	N-(2-{[2-(3,4-dimethylphenoxy)acetyl]amino}ethyl)nicotinamide	0±0.8
309	5-chloro-N-(3,4-dimethylphenyl)-2-methoxybenzamide	9±2.3
310	5-chloro-2-methoxy-N-(3-methoxyphenyl)benzamide	0±0.3
311	2-[(2-chloro-3-quinolinyl)methylene]quinuclidin-3-one	4±0.1
312	4-[(dimethylamino)sulfonyl]-N-[2-(4-ethylphenoxy)ethyl]benzamide	0±0.7
313	N-(4-ethoxyphenyl)-2-[(1-methyl-1H-tetraazol-5-yl)sulfanyl]propanamide	0±0.4
314	3-[2-(4-cyclohexylphenyl)-2-oxoethyl]-3-hydroxy-5-methyl-1,3-dihydro-2H-indol-2-one	0±1.2
315	2-amino-4-(2,5-dimethoxyphenyl)-6-(hydroxymethyl)-8-oxo-4,8-dihydropyrano[3,2-b]pyran-3-carbonitrile	0±3.8
316	6-(3,4-dimethoxyphenyl)-3-(2-fluorobenzyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	0±0.5
317	N-(4-acetylphenyl)-5-amino-7-thia-1,9-diazatetracyclo[9.2.2.0~2,10~.0~4,8~]pentadeca-2(10),3,5,8-tetraene-6-carboxamide	0±2.7
318	8-amino-N-(3,5-dichlorophenyl)-1,2,3,4-tetrahydro-1,4-ethanothieno[2,3-b][1,5]naphthyridine-7-carboxamide	3±1.3
319	N-(1H-tetraazol-5-yl)-N'-[3-(trifluoromethyl)phenyl]urea	2±1.1
320	4-[4-(3-methylphenyl)-1-piperazinyl]-2H-chromen-2-one	0±2.4

% CT, percent cytotoxicity±SD.

indicating its anticancer activity on cancer spheroids. On reviewing the literature, no biological activity at all was reported for this compound.

The spheroids of RPE1 normal human cell line have been previously shown to be completely nondividing [16], a state similar to most of adult normal tissues. This model was used to test the therapeutic window of SP1. The compound caused significant cytotoxicity (>59%) on RPE1 spheroids at doses from 12.5 to 50 μM (Fig. 4). However, at the dose 6.25 μM, it was totally safe on RPE1 spheroids and caused 21% cytotoxicity on MCF7 spheroids at the same concentration (Fig. 4). In the clonogenic study

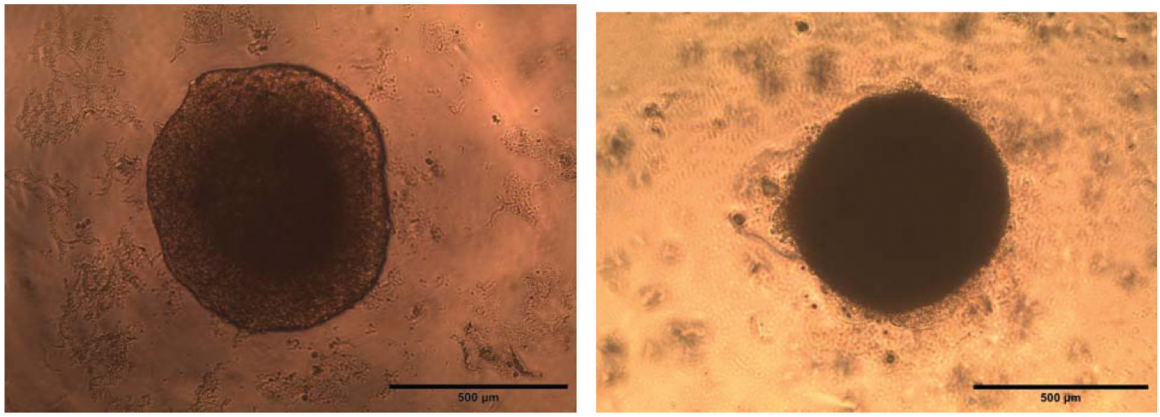
Figure 2



Compound SP1 (4,5-dibromo-6-oxo-1(6H)-pyridazinyl)methyl 3-chlorophenylcarbamate.

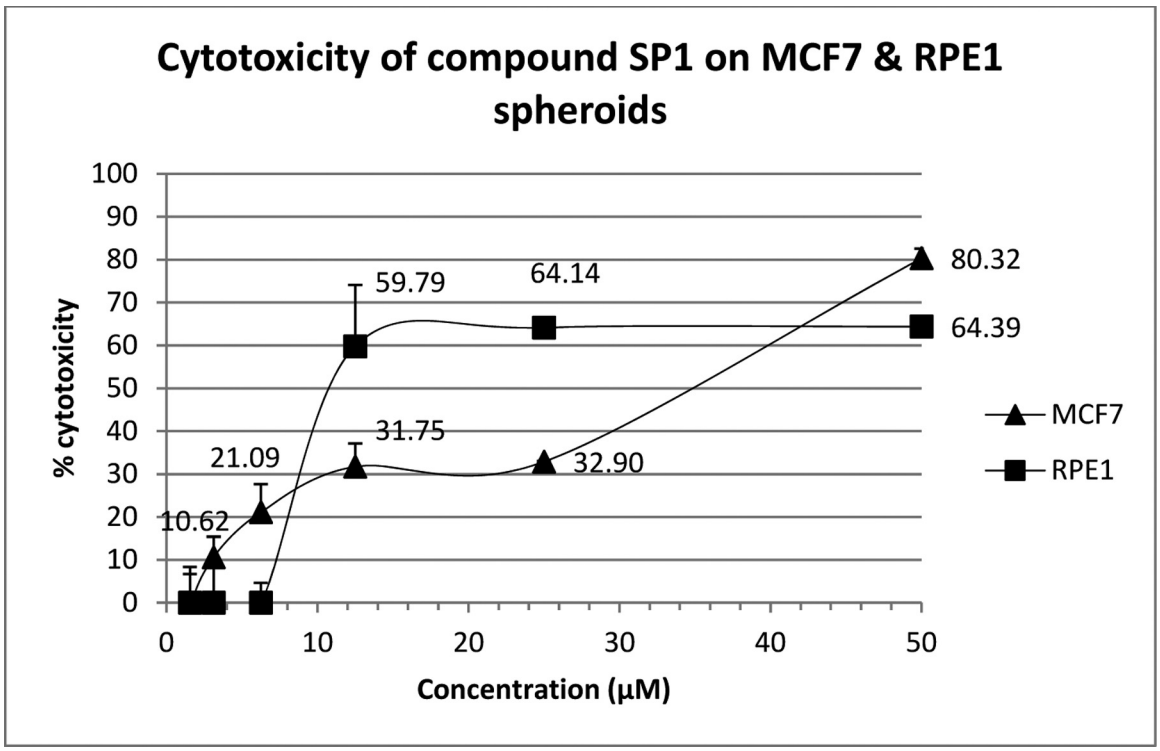
performed on the same two cell lines, a similar phenomenon was observed. SP1 caused high clonogenicity reduction in both cell line spheroids at concentrations from 12.5 to 50 µM, whereas was selectively toxic to cancer spheroids at concentration 6.25 µM (Fig. 5). Interestingly, the compound induced more than 99% reduction in clonogenicity at 6.25 µM, whereas scored 21% cytotoxicity at the same concentration on MCF7 spheroids. These results suggest that the residual living cells after the drug treatment (~80%) lost the clonogenic ability, possibly owing to senescence. Another possible explanation is that these residual cells die at longer

Figure 3



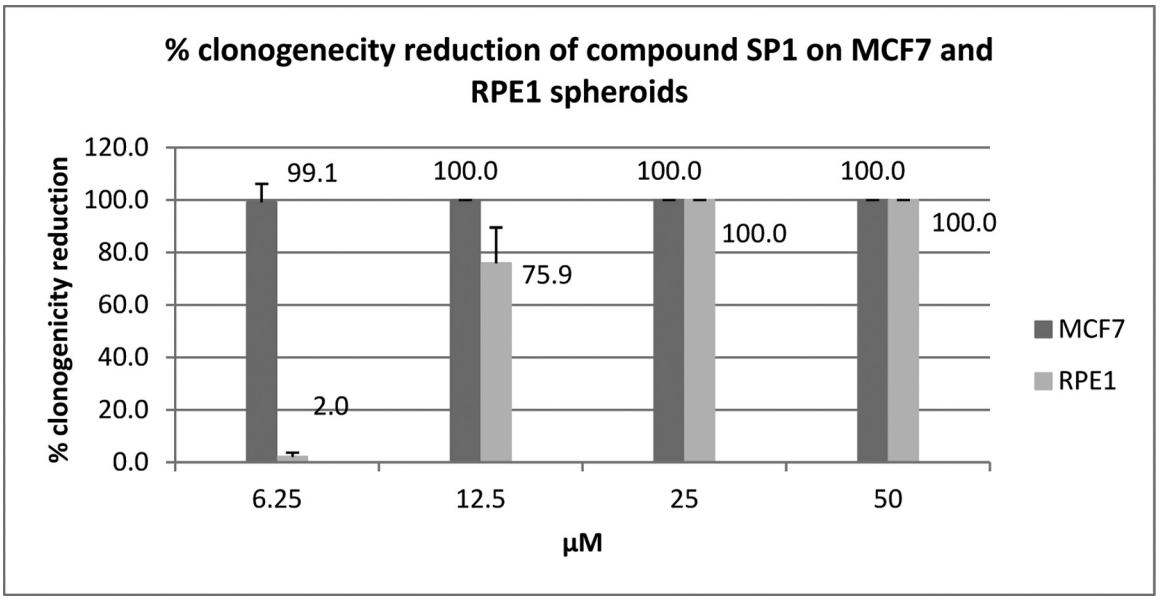
MCF7 and RPE1 spheroids at day 5 after seeding.

Figure 4



Dose–response results of compound SP1 on MCF7 and RPE1 spheroids.

Figure 5



Effect of the compound SP1 on clonogenicity of MCF7 and RPE1 spheroids.

time than 5 days after treatment, and thus were not clonogenic after seeding.

From these results, it can be concluded that although SP1 has minor activity on tumor tissue at low concentrations, at such concentrations, it is safe to normal cells. Thus, it can be anticipated that at multiple lower concentration treatments, a corresponding loss of regrowth of in-vivo tumors during recovery period can be expected after a chemotherapeutic dose of SP1 compound, which might lead to a successful remedy.

The results obtained both from the cytotoxicity and clonogenicity assays put the clonogenic assay in a significant position to correctly evaluate an anticancer agent and is recommended to be included in antitumor drug discovery procedures.

Screening of a large number of compounds on cancer and normal spheroids is highly encouraged. According to the author's point of view, this would most likely identify curative compounds that can be tolerated by patients with cancer.

Conclusion

The cytotoxicity profile of (4,5-dibromo-6-oxo-1 (6H)-pyridazinyl)methyl 3-chlorophenylcarbamate on both cancer and normal cell spheroids presents it as a promising anticancer compound that deserves further pharmacological and chemical studies.

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Conflicts of interest

There are no conflicts of interest.

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