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14. ABSTRACT BRCA2-Rad51 interaction is required for the Rad51-related DNA repair pathway. Thus, inhibition of their interaction is expected to sensitize tumor cells to certain DNA damaging agents. A panel of 14080 natural compounds from the Chinese National Center for Drug Screening has been partially screened using a yeast two-hybrid system utilizing specific Rad51/BRCA2 constructs. Growth of the Rad51/BRCA2 yeast strain in different media lead us to the selection of 20 candidate inhibitors for BRAC2-Rad51 interaction. Three of these compounds present minimal toxicity to the yeast strains respect to their specific inhibitory activity and thus are the first candidates to be tested for their ability to sensitize breast cancer cells to cisplatin.					
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A. SUMMARY OF THE PROJECT

BACKGROUND

BRCA2 directly interacts with Rad51 promoting Rad51 directed DNA repair. Repair of interstrand crosslinks induced by interstrand crosslinking agents, involves the Rad51 related homologous recombinational repair pathway (HRR) (1). The homologous recombinational repair process requires the assembly of multienzymatic complexes visualized immunocytochemically as Rad51 nuclear foci. These complexes include the Rad51 paralogs family members such as (Rad51, Rad52, Rad54, Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3) and the breast cancer associated proteins, BRCA1 and BRCA2. Defective cell lines in each of the above mentioned proteins present similar phenotypes: spontaneous chromosomal aberrations, high sensitivity to killing by cross-linking agents, mild sensitivity to gamma rays and attenuated Rad51 focus formation after exposure to ionizing radiation (2,3). Our innovative contribution will be to find natural compounds to sensitize tumor cells to chemotherapeutic agents by inhibiting BRCA2/Rad51 interaction.

RATIONALE

BRCA2 is central to HRR repair through BRC-mediated Rad51 interactions required for the assembly of DNA damage-induced RAD51 foci. Inhibition of their interaction would sensitize tumor cells to DNA crosslinking agents. Therefore our drug discovery program focus on the identification of compounds capable of competitively block the interaction BRAC2 and Rad51 (3).

OBJECTIVE

Find natural compounds that will inhibit BRCA2-Rad51 interaction in order to inhibit the homologous recombinational process and consequently sensitize breast tumor cells to therapeutic agents.

STRATEGY

The panel of 10000 natural compounds from the Chinese National Center for Drug Screening is being screened using a yeast two hybrid system utilizing the Rad51/BRCA2 constructs (4). Compounds that specifically inhibit the Rad51-BRCA2 interaction will be further tested as indicated below.

BIOLOGICAL EVALUATION

The biological activity of selected compounds using the two yeast hybrid system will be tested in a sporadic human breast cancer cell line panel (expressing wild type BRCA2) using the NCI sulthorodamine B assay (5). All tests will be performed using sublethal doses of the selected compounds, in combination with the IC₂₀ and IC₅₀ concentration of cisplatin. Drug interactions (antagonism, additive, or synergism) will be determined (6). The ability of to alter homologous recombinational repair will be examined immunocytochemically looking at changes on cisplatin-induced Rad51 foci in the presence of selected compounds. Disruption of Rad51-BRCA2 interaction will be confirmed by cross immunoprecipitation followed by western blot analysis using specific antibodies (4).

B. ORIGINAL STATEMENT OF WORK

Task # 1 Construction of the BRCA2-Rad51 Yeast 2 Hybrid System

STATUS: COMPLETED

Task #2 Screening of candidate compounds to inhibit BRCA2-RAD51 interaction

STATUS: IN PROGRESS

Task #3 Biological Evaluation of candidate inhibitors of BRCA2-Rad51 interaction

STATUS: PENDING

C. ANNUAL PORGRESS REPORT

C.1. Task # 1 Construction of the BRCA2-Rad51 Yeast 2 Hybrid System

STATUS: COMPLETED

The BRCA2 domain which interacts with Rad51, was cloned from a human cDNA library using specific primers for human wild type BRCA2 (Accession Number NM_000059). The cDNA sequence corresponding to BRCA2 3196-3991 was amplified by PCR and subcloned into the pBTM116 vector in frame to LexA (BRCA2-LexA hereafter)(6).

The full length Rad51 was cloned from a human cDNA library using specific primers for human wild type Rad51 (Accession Number NM_002875). The Rad51 full cDNA was amplified by PCR and subcloned into the plasmid PACT2 vector in frame to GAL4-TA (GALA4-TA-Rad51 hereafter)(7).

We confirm the proper orientation of the inserts and sequence by agarose electrophoresis after restriction endonuclease digestion and sequencing respectively.

The vectors were transformed either alone or together into the yeast strain L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) as shown in **Table 1**. The L40 strain can't growth in medium lacking the amino acids leu, trp and his. After transformation the yeast were grown in selective medium (SD) lacking the amino acids leu, trp and his. BRCA2-LexA or GALA4-TA-Rad51 transformed yeast were able to growth in medium containing His but lacking Trp or Leu respectively. Only yeast transformed with both vectors in which the fusion proteins interact was able to growth in SD media lacking the three amino acids (Leu, Trp and His)(7).

Table 1

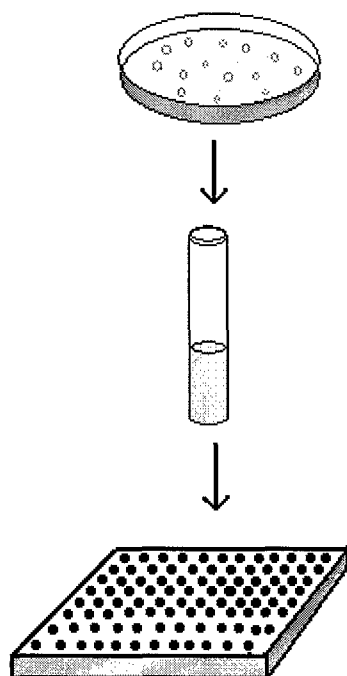
Yeast Transformation	Growth	Growth Tested in SD-LTH agar plates: yeast nitrogen base without amino acids without Leu, Trp and His, supplemented with dextrose and 3-AT*
pBTM116-BRCA2 vector	Negative	
pPACT2-Rad51 vector	Negative	
pBTM116-BRCA2 + pPACT2 vectors	Negative	
pBTM116 + pPACT2-Rad51 vectors	Negative	
pBTM116-BRCA2+ pPACT2-Rad51 vectors	Positive	

*3-AT, 3-Amino-Triazol is used to inhibit the basal expression of *His3* to avoid the growth of false positive yeast colonies.

C.2. Task #2 Screening of candidate compounds to inhibit BRCA2-RAD51 Interaction.

STATUS: IN PROGRESS

C.2.1.Primary Screening strategy



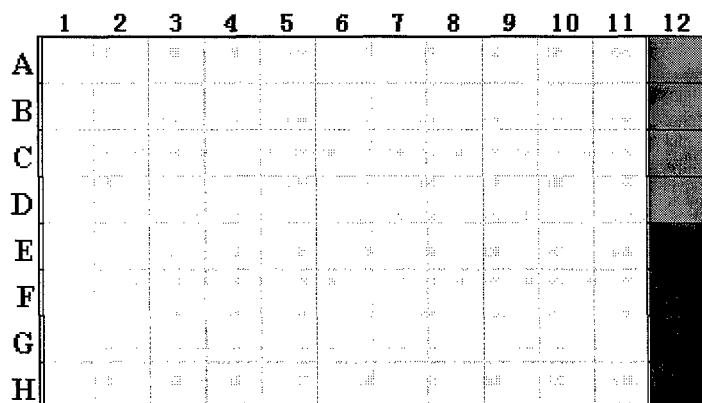
After transfection with the pBTM116-BRCA2 and the pPACT2-Rad51 vectors, the L40 yeast were plated on SD/-Leu/-Trp/-His/ (1mM 3-AT) to select for colonies, in which BRCA2 and RAD51 interact.

The clones were inoculated and grown to the mid-log phase in SD/-Leu/-Trp/-His/ (1mM 3-AT).

The cultures were diluted about 40 folds and transferred to each well (200 μ l) of the 96 well plate, containing has 2 μ l of a compound (1mg/ml) in 100% DMSO.

The plates were incubated overnight then read at 600nm in a microplatereader to determine the grow status.

96 well Plate format



 High control(2 μ l DMSO)

 Low control(2 μ l Amphotericin B, final conc. 5 μ g/ml)

 Compound(2 μ l compounds in 100% DMSO)

(DMSO is the vehicle used to dissolve the compounds and Amphotericin B is an anti-yeast agent).

Using this format, the activity of 80 compounds can be obtained from one 96 well plate.

If a given compound inhibits the interaction between BRCA2 and Rad51, the growth of the yeast is inhibited due to the lack of His in the medium. The growth inhibition is calculated respect to the growth in the presence of vehicle (2 μ l DMSO).

C.2.2.Primary screening results

The primary screening was carried out as described in C.2.1. with 14,080 compounds, and the final concentration of each compound was 10 μ g/ml in SD/-Leu/-Trp/-His/ (1mM 3-AT) media.

The percentage of inhibition and the distribution rate of inhibition obtained are shown in **Table 2**.

Table 2. Inhibition rate

% of Inhibition	≥ 80	≥ 85	≥ 90	≥ 95
Hits	259	171	114	73
Hit rate	1.84%	1.21%	0.81%	0.52%

C.2.3. Secondary screening strategy

The compounds (Hits) that showed $\geq 80\%$ of inhibition of BRCA2 and RAD51 interaction during the primary screening (259 in total, **Table 2**) were chosen for further analyses. Sister cultures were grown in the presence of 10 $\mu\text{g/ml}$ of each compound plus SD/-Leu/-Trp/-His/ (1mM 3-AT) or SD/-Leu/-Trp. Inhibition of growth in the first medium (without Leu, Trp and His) indicated specific inhibition of BRCA2-Rad51 interaction while inhibition of growth in the second medium (without Leu and Trp) indicated toxicity.

From the 259 compounds, 130 have been already tested using the secondary screening, 20 of which showed selective growth inhibition in medium lacking Leu, Trp and His respect to the medium lacking Leu and Trp (**Table 3**).

Table 3. Inhibition rate comparison.

	SD/-LTH	SD/-LT(+His)
	Inhibition%	
1	91.2	23.3
2	80.6	36.0
3	88.6	44.1
4	84.8	52.1
5	80.1	19.5
6	80.1	33.8
7	80.8	-7.0
8	80.1	37.4
9	96.8	40.1
10	80.1	35.6
11	89.0	-3.0
12	91.7	27.6
13	96.8	30.9
14	89.6	-3.1
15	91.7	30.2
16	97.7	45.2
17	80.4	35.0
18	91.8	18.9
19	86.7	9.5
20	94.3	38.5

C.2.4 IC₅₀ value of inhibitor candidates

IC₅₀ determination was made for the 20 compounds that showed selectivity in the secondary screening. From a serial dilution of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 $\mu\text{g/ml}$, 18 compounds were found to have IC₅₀ values less than 10 $\mu\text{g/ml}$ in SD/-Leu/-Trp/-His/ (1mM 3-AT) (**Table 4**).

Table 4. IC₅₀ value.

	SD/-LTH	SD/-LT(+his)	SD/-LTH
	%Inhibition		IC ₅₀ (µg/ml)
1	91.2	23.3	6.91
2	80.6	36.0	8.67
3	88.6	44.1	7.29
4	84.8	52.1	4.21
5	80.1	19.5	5.51
6	80.1	33.8	5.34
7	80.8	-7.0	3.33
8	80.1	37.4	2.06
9	96.8	40.1	2.88
10	80.1	35.6	>10
11	89.0	-3.0	>10
12	91.7	27.6	2.69
13	96.8	30.9	1.07
14	89.6	-3.1	3.77
15	91.7	30.2	2.46
16	97.7	45.2	2.53
17	80.4	35.0	4.27
18	91.8	18.9	8.09
19	86.7	9.5	6.84
20	94.3	38.5	6.90

In bold are indicated the best and first candidates to be tested to sensitize breast cancer cells to cisplatin as described in project summary (**BIOLOGICAL EVALUATION**).

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