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Synthesis and Biological Evaluation of DIDS Analogues as Efficient Inhibitors of RAD51 Involved in Homologous Recombination

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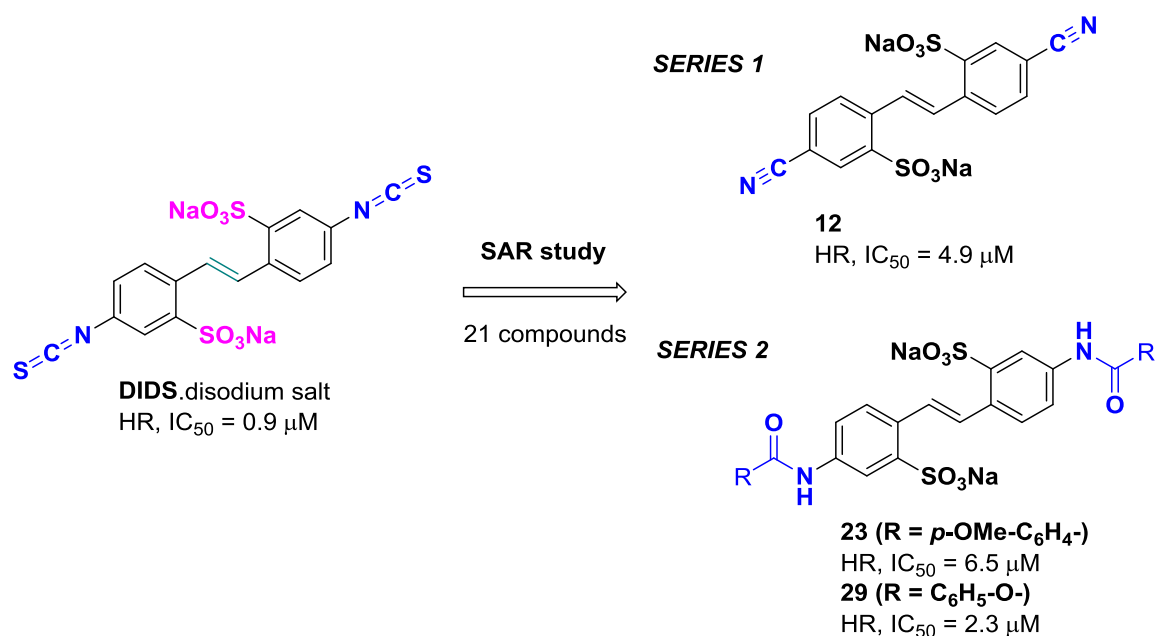
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Abstract

RAD51 is a pivotal protein of the homologous recombination DNA repair pathway, and is overexpressed in some cancer cells, disrupting then the efficiency of cancer-treatments. The development of RAD51 inhibitors appears as a promising solution to restore these cancer cells sensitization to radio- or chemotherapy. From a small molecule identified as a modulator of RAD51, the 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), two series of analogues with small or bulky substituents on the aromatic parts of the stilbene moiety were prepared for a structure-activity relationship study. Three compounds, the cyano analogue (**12**), and benzamide (**23**) or phenylcarbamate (**29**) analogues of DIDS were characterized as novel potent RAD51 inhibitors with HR inhibition in the micromolar range.

Graphical abstract



Key words : Homologous recombination, DNA repair, RAD51, stilbene 2,2'-disulfonic compounds, inhibitor.

DNA repair process is particularly important in maintaining genomic integrity. In proliferative diseases therapies such as radiotherapy or chemotherapy, this process can restore the DNA damaged with the treatment, and induce a radio- or chemo-resistance. Homologous recombination (HR) is one of the two cellular pathways that repair DNA double-strand breaks (DSBs) [1]. It is catalyzed with RAD51 a highly conserved protein overexpressed in a wide variety of cancers and consequently contributing to drug resistance [2]. Elevated level of RAD51 mRNA is also associated with higher risk of tumor relapse and metastases [3]. It is to be noted that currently cancer treatment for patients with deficiency in HR-DNA repair systems are accessible with small molecules such as PARP inhibitors [4]. RAD51 is also investigated for its clinical utility as a biomarker [5] for the selection of patients with HR deficient tumors [6] or for prognosis of cellular DNA damage induced by chemotherapy [7]. RAD51 disrupting DNA repair by HR either indirectly or directly is also clinically relevant especially to improve response to radio- or chemotherapy treatment.

During the last decade, extended studies have been done with RAD51 inhibitors including peptides, nucleic acid and small synthetic molecules. Peptide inhibitors have been generated to modulate the interaction between RAD51 monomer-monomer. Several peptide sequences from BRC motifs of BRCA2 have shown efficiency to dissociate the nucleofilament of RAD51 by inhibiting the auto-association of RAD51 on the DNA.[8] DNA aptamers (A13, 130 or A79) were identified by Martinez and collaborators as direct modulators of RAD51 and their inhibitory effect impacted nucleofilament formation rather than dissociation, inducing so the stability of ATP/RAD51/ssDNA complex [9]. Small molecular weight molecules are also required to interfere with RAD51 protein to disrupt either the polymerization and heptamer formation process or the binding of RAD51 to ssDNA during nucleofilament formation [1] [10]. Most of these molecules have been identified by high throughput screening of chemical compounds libraries. From the NIH Small Molecule Repository (>20 000 compounds), Fei Huang and collaborators [11] identified a compound B02 (Figure 1, compound 1) which selectively inhibited hRAD51 with an IC₅₀ of 27.4 μM on a D-loop assay. They recently published a series of B02 isomers with significant higher efficiency in inhibition of HR in human cells (Figure 1, compound 2). [12] Mediated by DNA recombinase RAD51, B02 was also proved to sensitize malaria parasites to chemotherapeutics

such as artemisinin and chloroquine [13]. With a similar virtual approach Connell and collaborators identified RI-1 (Figure 1, compound **3**) that disrupted HR with an IC_{50} of 20 μ M. Due to its reactivity as a Michael acceptor [14] inducing an irreversible profile, chemical optimization of RI-1 was done and substitution of the chloro substituent with a p-methoxyphenyl group afforded a compound **4** (Figure 1, RI-2) with lower activity but reversible binding.[15] The same authors applied two parallel screening of more than 8,000 compounds on RAD51-ssDNA binding assay and RAD51-dependent D-loop assay, in order to identify selective inhibitors. After chemical optimization of a promising lead, compound **5** (Figure 1, RI (dl)-2) was found to displayed improved D-loop activity, without detectable effect on RAD51-ssDNA binding.[16] From Nanosyn libraries (24,000 compounds) a sulfonamide synthetic alkaloid **6** (Figure 1, IBR2) was confirmed by SPR to bind RAD51 and to inhibit HR by disrupting RAD51 heptamers inducing filament formation.[17] From a library of 1120 compounds, Chicago Sky Blue (CSB), a dye molecule was revealed as RAD51 inhibitor in the low nanomolar range (Figure 1, compound **7**).[18] Screening 185 known biological compounds and drugs, on RAD51 strand-exchange assay *in vitro*, Kurumizaka's team identified a disulfonic compound 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid **8** (DIDS), with 50 % effect around 1-2 μ M.[19]

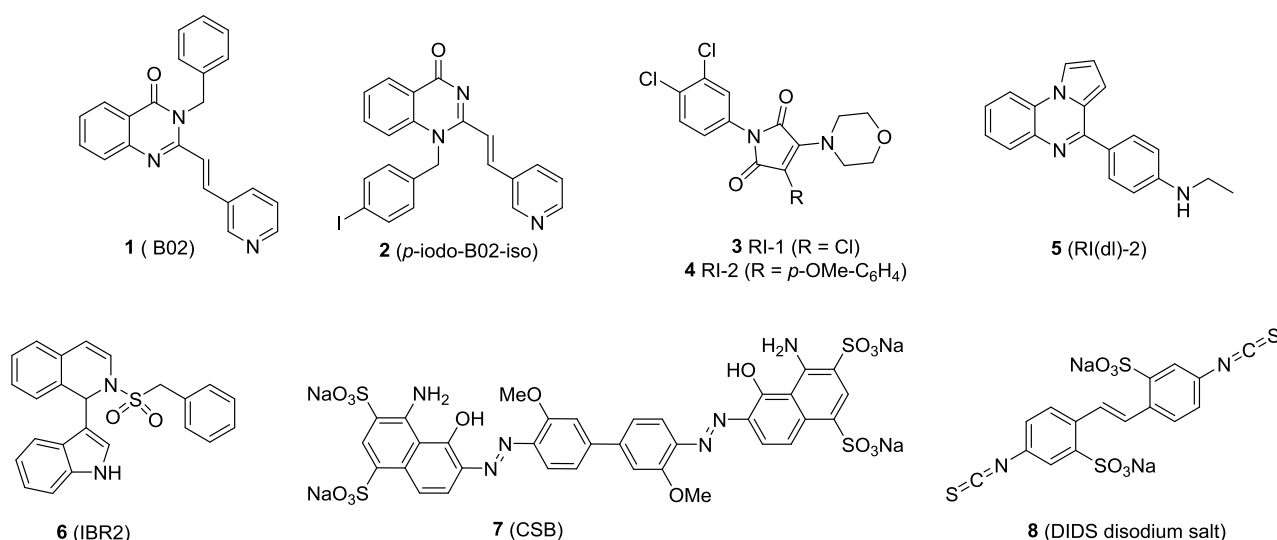


Fig 1. Small (hetero)aromatic molecules, modulators of the HR process.

Although it seemed that RAD51 inhibition resulted from DIDS direct interaction with RAD51 protein, DIDS mechanism of action was not clearly elucidated. Moreover, DIDS

suffered from limitations which impair its potential use against cancer, regarding for example selectivity over other biological targets or chemical stability of the molecule. Especially, DIDS as inhibitor is not specific of the RAD51 protein. It also inhibits membrane transporters [20] or anion channels [21], and its isothiocyanate functions may be responsible of some of these activities. [22] As a chemical weakness, DIDS stability in water has been discussed [23], and the potential hydrolysis of the isothiocyanate function could afford DIDS oligomers also identified as chloride channel blockers. [24]

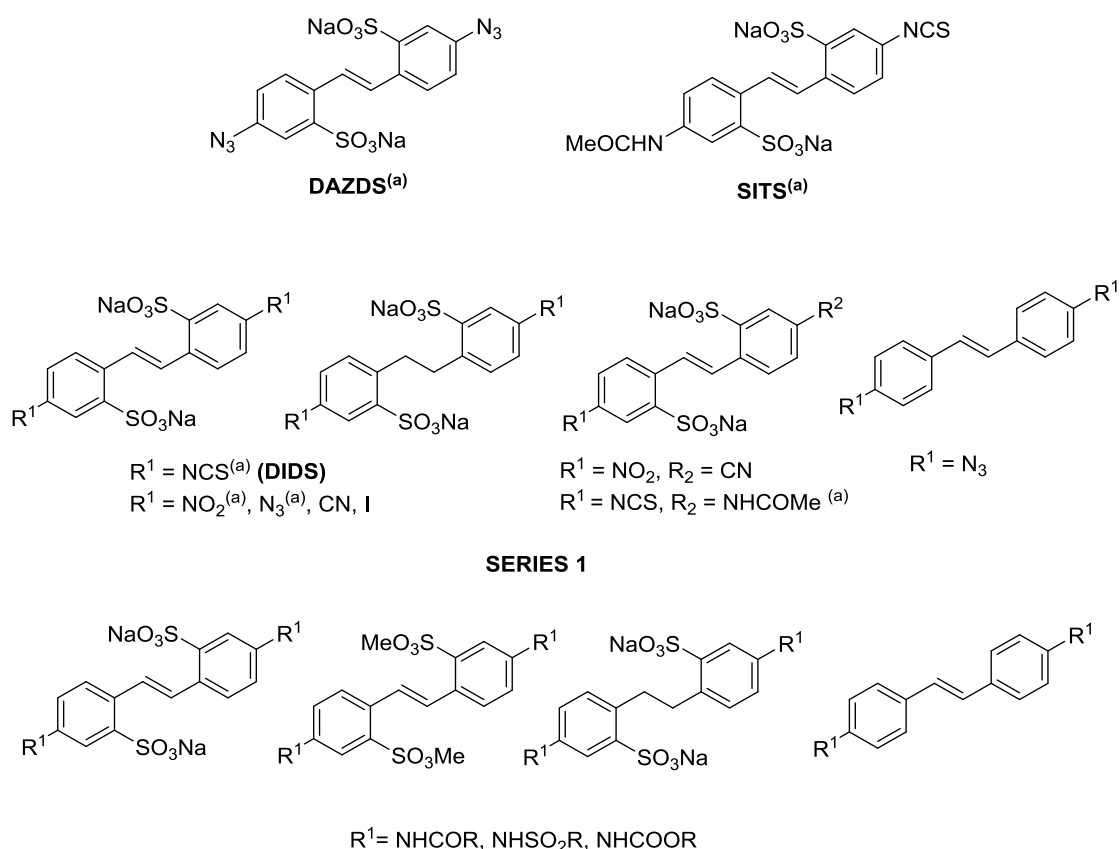


Fig. 2 : Commercial ^(a) and synthetic DIDS analogues studied as RAD51 potential inhibitors.

In a previous work, we selected four commercial analogues of DIDS and showed that DAZDS and SITS (Figure 2) were also able to prevent the formation of D-loop with an IC₅₀ of 44.3 μM and 29 μM respectively, compared to 0.9 μM for the DIDS [25].

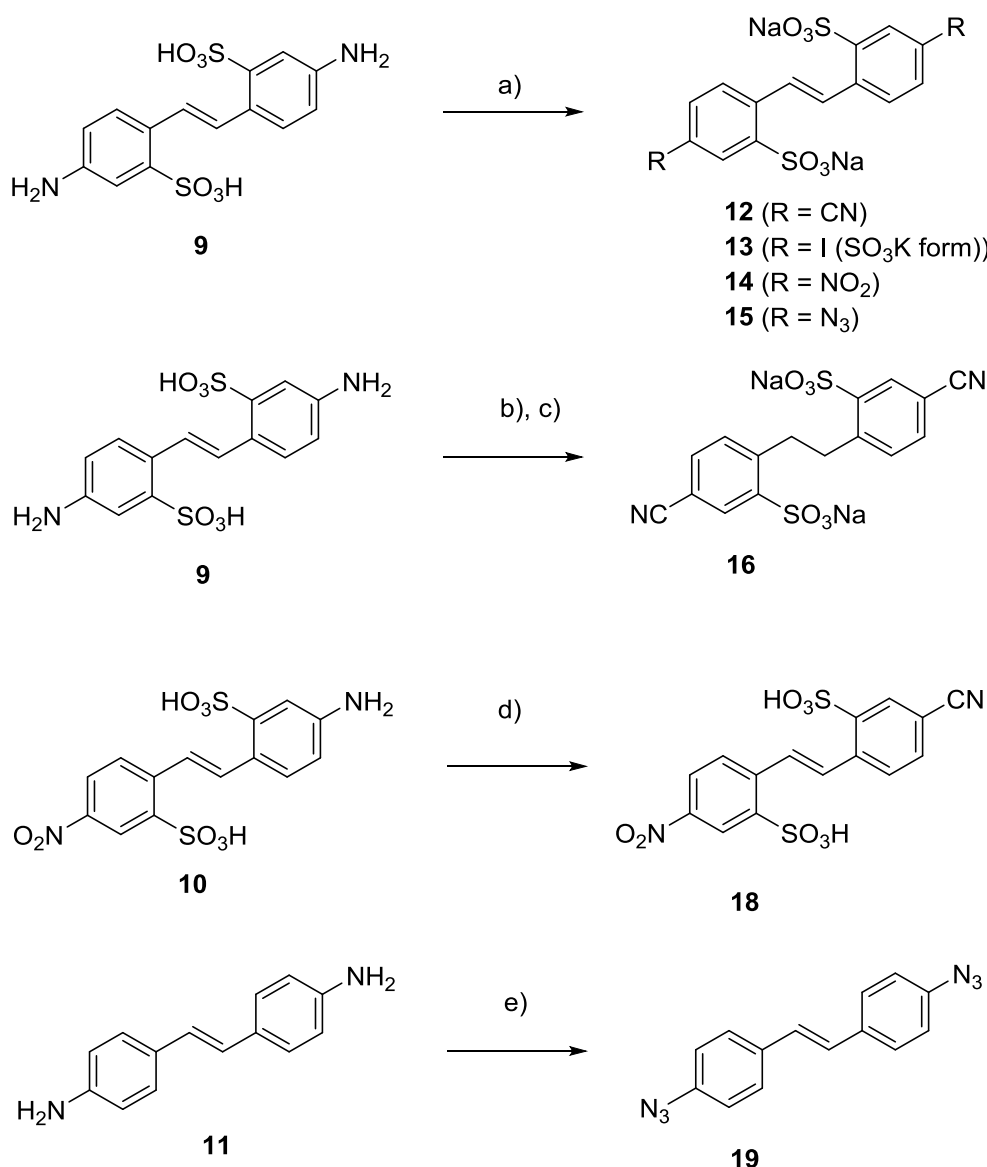
In this work, we have performed the synthesis of two novel series of DIDS analogues as potential RAD51 inhibitors. Thereby chemical modifications were introduced on three

distinct positions of the 4,4'-substituted-2,2'-disulfonic stilbene scaffold for a structure-activity relationship study, in order to identify a good substitute to DIDS.

The compounds were tested *in vitro*, firstly for their effect on the invasion DNA (D-loop assay) and DNA strand exchange steps of RAD51 activity and secondly for their cytotoxicity effect on cancer cell lines overexpressing RAD51.

Two series of DIDS analogues have been synthesized, the first one including compounds bearing small chemical groups in place of the isothiocyanate group (NCS) in DIDS (Figure 2, series 1) and the second one derived from 4,4'-diamino analogue of DIDS (DADS) to develop compounds such as amide, sulfonamide and carbamate analogues of DIDS (Figure 2, series 2). These functions are known to be chemically stable in neutral conditions compared to the isothiocyanate one and nitrile or carbamate groups are also key structural motifs of many natural or synthetic drugs.[26, 27] To complete the SAR study, molecules with methyl sulfonate ester function or lacking the sulfonic acid function as well as the unsaturated ethylenic chain, have also been prepared, in order to determine the impact of these modifications on the HR activity. These novel DIDS analogues were prepared from commercial compounds such as 4,4'-diamino-2,2'-stilbenedisulfonic acid **9** (DADS), its 4-nitro-4'-amino analogue **10** and the 4,4'-diamino stilbene **11**.

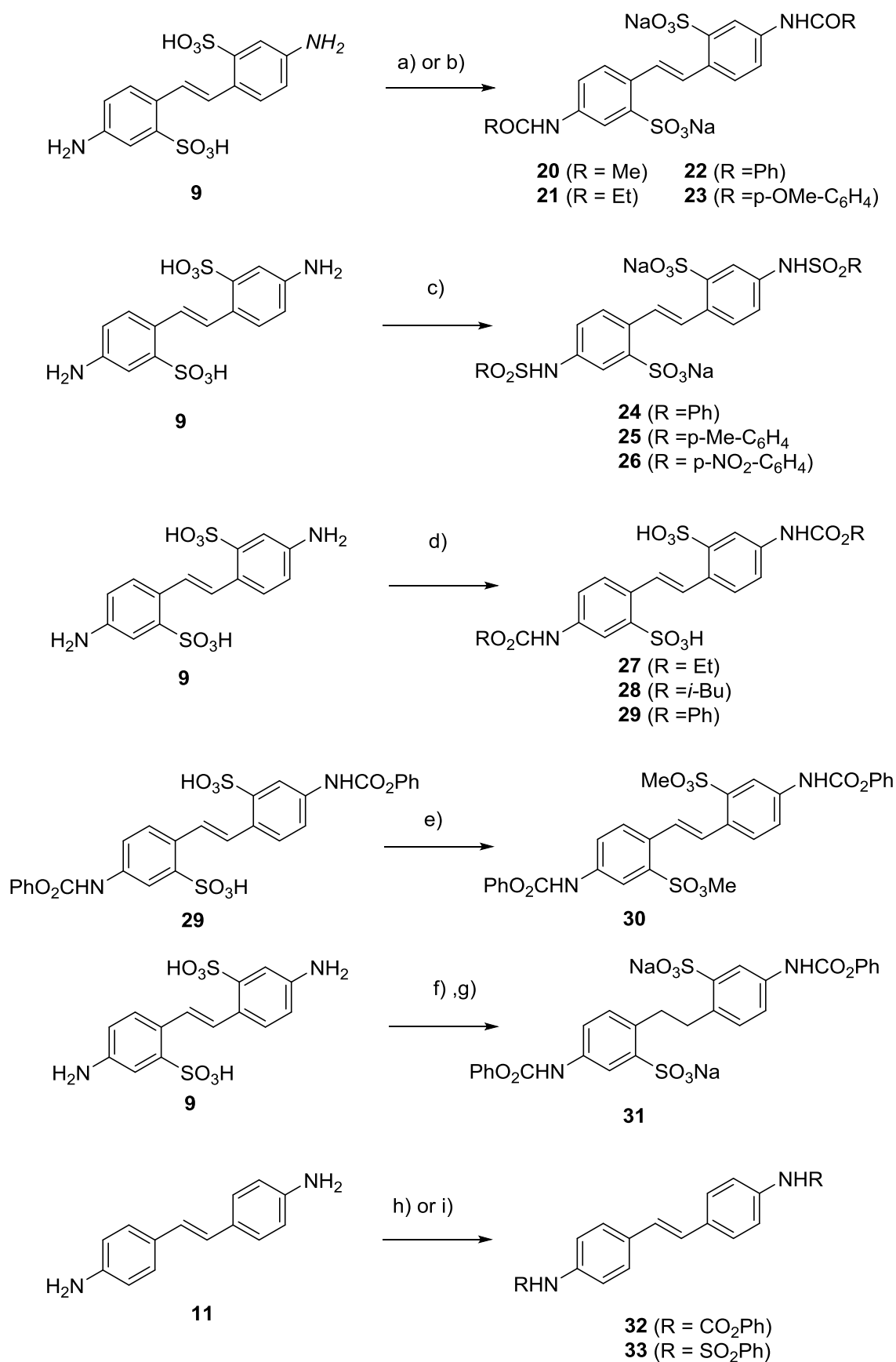
Starting from diamino or monoamino stilbene compounds **9**, **10** or **11**, small substituents such as cyano, iodo or azido substituents could be efficiently introduced using classical Sandmeyer conditions (Scheme 1). The targeted compounds **12-13**, **18** and **19** were obtained with moderate to good yield (44 to 90 %) after purification by simple precipitation in a mixture of MeOH/Et₂O. To access to the unsaturated cyano analogue **16**, reduction of the double bond of DADS to give the corresponding 4,4'-diaminodibenzyl-2,2'-disulfonic acid (DADB) was performed in basic conditions using Raney Nickel as the catalyst [28], prior to the Sandmeyer reaction.



Scheme 1. Synthesis of DIDS analogues of series 1 : Reagents and conditions: a) NaNO₂ (2 equiv), AcOH (5 equiv), NaCN (4 equiv), CuCl (0.25 equiv), H₂O, rt, 2 d, 67% for **12**; NaNO₂ (2 equiv), AcOH (5 equiv), KI (5 equiv), CuI (0.1 equiv), H₂O, rt, 2 d, 47% for **13**; **14** and **15** are commercial compounds; b) RaNi, [H₂] 1 atm., NaOH (2 equiv), H₂O, 50 °C, 2 d, 74% of DADB; c) DADB, NaNO₂ (2 equiv), AcOH (5 equiv), NaCN (4 equiv), CuCl (0.25 equiv) water, rt, 3 d, 44% for **16**; d) NaNO₂ (1 equiv), AcOH (3 equiv), NaCN (2 equiv), CuCl (0.5 equiv), H₂O, rt, 1.5 d, 71%; e) NaNO₂ (2 equiv), AcOH (5 equiv), NaN₃ (2 equiv), H₂O, rt, 3 h, 90% .

The second series of DIDS analogues, compounds **20-29** and **32-33**, was prepared by acylation or sulfonylation of the amino precursors **9** or **11** (Scheme 2). In basic aqueous conditions using anhydrides, sulfonyl chlorides or chloroformates as the reagents, the amides **20-22**, the sulfonamides **24-26** or the carbamates **27-29** were obtained with good yields (62 to 85 %) after purification by simple precipitation. With acyl chlorides as the acylating agents, reactions were performed in DMF using triethylamine as the base, and the amide **23** was

obtained with a moderate yield of 44 %. To determine the rule of the sulfonic function on the activity profile, esterification of the phenyl carbamate analogue **29** was achieved by refluxing its acidic form in toluene in the presence of an excess of trimethyl orthoacetate.[29] [30] The methyl sulfonate derivative **30** was obtained with 32 % yield. The sodium salt of DADB (reduced DADS) was also acylated with an excess of phenyl chloroformate in aqueous medium (H₂O/dioxane) to give compound **31** with 58 % yield. It is to be noted that some attempt to reduce the double bond of compound **29** with NaBH₄ and Raney Nickel [31] to afford **31** in a single step, didn't work. Finally diamino stilbene **11** was reacted with phenyl chloroformate or phenyl sulfonyl chloride in dichloromethane, in the presence of an organic base, to give respectively carbamate **32** (54 % yield) and sulfonamide **33** (41 % yield).



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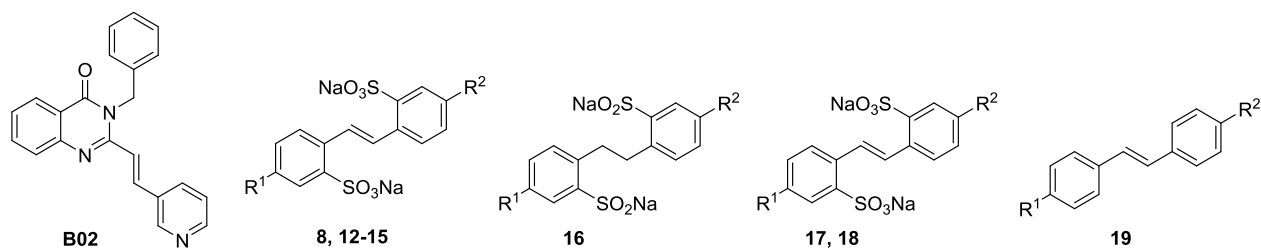
157 **Scheme 2. Synthesis of DIDS analogues of series 2** : Reagents and conditions: a)
 158 ROCOCOR (4 eq.), Na₂CO₃ (5 eq.), nBu₄NCl (0.1 eq.), H₂O/dioxane (5:1), 2h, 62 % for **20**

(R = Me); overnight, 76 % for **21** (R = Et); overnight, 70 % for **22** (R = C₆H₅); b) RCOCl (4 eq.), DIEA (5 eq.), DMF, 80 °C, 4 h, 44 % for **23** (R = p-OMe-C₆H₄); c) RSO₂Cl (2.5 eq.), Na₂CO₃ (5 eq.), H₂O/dioxane (5:1), rt, overnight, 61 % for **24** (R = C₆H₅); 87 % for **25** (R = p-Me-C₆H₄); 81 % for **26** (R = p-NO₂-C₆H₄); d) ROCOCl (2.5 eq.), Na₂CO₃ (5 eq.), H₂O/dioxane (5:1), rt, overnight, 66 % for **27** (R = Et); 67 % for **28** (R = *iso*Bu); 90 % for **29** (R = C₆H₅); e) CH₃C(OMe)₃ (4 eq.), toluene, reflux, 17 h, 32 %; f) Ra-Ni, [H₂] 1 atm., NaOH (2 equiv), H₂O, 50 °C, 2 d, 74% of DADB; g) PhOCOCl (4 eq.), H₂O/dioxane (3:1), overnight, 58 %; h) PhOCOCl (4 eq.), DIEA (6 eq.), CH₂Cl₂, rt, 24 h, 54 % for **32**; i) RSO₂Cl (2.2 eq.), Et₃N (4 eq.), CH₂Cl₂, rt, 24 h, 41 % for **33**.

With these as-prepared DIDS analogues in hands, we next focused our attention to evaluate them as potential RAD51 inhibitors with two *in vitro* activity tests developed to identify strand exchange activity. The first bioassay is an electrophoresis-based DNA strand exchange [18] between a labeled short single strand ssDNA (58-ssDNA*) and a homologous double strand ds-DNA (33-ds-DNA) catalyzed by RAD51. Using polyacrylamide gel electrophoresis the formation of a novel dsDNA formed (58/33-dsDNA*) could be measured in a dose-dependent manner. The second bioassay is a DNA displacement loop called D-loop [32] using labeled 100-ss-DNA**.

The compounds of series 1 and series 2 were evaluated in one or both biochemical assays and compared to DIDS which gave similar results in the electrophoresis-based DNA exchange assay or the D-loop assay, and IC₅₀ values of 0.75 μM and 0.9 μM respectively. We could observe that substitution of the isothiocyanate function (NCS) with a strongly electro-withdrawing nitro group (compound **14**) dramatically decreased the effect up to 50 fold. A bulky polarizing substituent such as a iodo substituent (compound **13**, IC₅₀ = 34 μM) as well as the azido function (compound **15**, IC₅₀ = 21.5 μM) were less deleterious, but the sulfonate functions seemed to be crucial as the activity of the azido compound **19** devoid of these functions decreased with an IC₅₀ value up to 50 μM. Our best compound in this first series was compound **12**, with cyano substituents in place of the isothiocyanate ones and giving RAD51 activity inhibition with an IC₅₀ value of 4.9 μM. Furthermore in this series, unsymmetrical DIDS analogues bearing either one NCS group such as the commercial acetamido compound **17**, or one CN function such as the nitro compound **18** conserved RAD51 activity, but in the range of 30 micromolar, six fold higher than our best di-cyano compound **12**. Finally reduction of the double bond of dicyano compound **12**, to give the flexible 1,2-diphenyl ethane analogue **16**, induced loss of RAD51 activity.

Table 1 : RAD51 activity evaluation for compounds of series 1



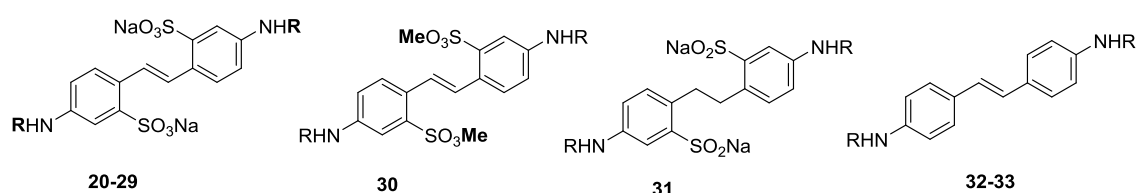
Compound	R ¹	R ²	IC ₅₀ (μM) (DNA strand exchange assay)	IC ₅₀ (μM) (D-loop)
B02	-	-	-	27.4 ^b
8^a (DIDS)	NCS	NCS	0.75	0.9
12	CN	CN	1.3	4.9
13	I	I	17.5	34
14^a	NO ₂	NO ₂	-	>50
15^a (DAZDS)	N ₃	N ₃	10	21.5
16	CN	CN	-	>50
17^a (SITS)	NCS	NHCOMe	-	29
18	CN	NO ₂	-	20
19	N ₃	N ₃	-	>50

^{a)} commercial compound; ^{b)} ref 11

For the second series with bulky amido, sulfonamide or carbonate residues in place of the NCS one, only the residues with aromatic moieties, such as compounds **23**, **25**, **29**, **31** and **32**, gave IC₅₀ values in the range of 2.3 μM to 30 μM. Sulfonamide compounds **24-26**, with an acidic NH proton, were less efficient than amide (compound **23**) or carbonate (compound **29**) analogues. The best compound in this second series was the diphenylcarbonate **29**, giving similar results in the electrophoresis-based DNA exchange assay and the D-loop assay, with IC₅₀ values of 3.8 μM and 2.3 μM respectively. We wanted to block the ionic character of compound **29**, by esterification of the sulfonic functions. Satisfyingly we observed that the bis-methyl sulfonate **30** was inactive, pointing out the crucial role of these free sulfonic functions to block HR activity. Here also, reduction of the double bond to access to compound **31**, or exclusion of the sulfonic functions (compound **32**) induced a 10-fold decrease of the activity value, compared to the parent compound **29**.

If the double bond as well as the sulfonic functions of these stilbene compounds appeared as important elements for RAD51 activity, complementary structure-activity conclusions still remain difficult. Taking in account that the cyano functions in compound **12** from series 1, and the benzamide or phenylcarbamate groups of compounds **23** and **29** from series 2, have no similar physico-chemical properties, this could imply that each series has its specific interactions in a same binding site, or that each series has its own binding site with RAD51.

Table 2 : RAD51 activity evaluation for compounds of series 2



Compound	R	IC ₅₀ (μM) (DNA strand exchange assay)	IC ₅₀ (μM) (D-loop)
8 (DIDS)	-	0.75	0.9
20	MeCO-	>50	-
21	EtCO-	>50	-

22	PhCO-	>50	-
23	<i>p</i> OMe-C ₆ H ₄ CO-	3.0	6.5
24	PhSO ₂ -	-	41.3
25	<i>p</i> Me-C ₆ H ₄ SO ₂ -	-	29
26	<i>p</i> NO ₂ -C ₆ H ₄ SO ₂ -	-	>50
27	EtOCO-	>50	-
28	<i>i</i> BuOCO-	-	>50
29	PhOCO-	3.8	2.3
30	PhOCO-	-	>50
31	PhOCO-	-	≈ 30
32	PhOCO-	-	30.5
33	PhSO ₂ -	-	>50

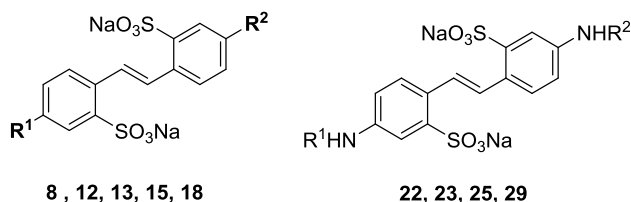
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224 To envisage *in cellulo* evaluation of this family of molecules, the most interesting compounds
225 were selected and their cytotoxicity was evaluated on prostatic cancer cell (DU145) by the
226 MTT assay after 72h of exposure at 1μM, 10μM and 50μM (Table 3). The known
227 quinazolinone compound **B02**, showed a highly cytotoxic effect on DU145, reducing the
228 viability of DU145 from 21% to 91%, at the concentrations of 10μM and 50 μM,
229 respectively. In addition, DIDS poorly reduced cell viability from 10 to 17% in a dose-
230 dependent way. We could observe that most of the selected DIDS analogues of series 1 (**12**,
231 **13**, **15**, **18**) or of series 2 (**22**, **23**, **25**, **29**) had very low or no cytotoxic effect on DU145 cells

even at the high concentration of 50 μ M. Particularly, our most potent analogues such as di-cyano compound **12**, and di-phenylcarbamate compound **29**, totally preserved cell viability at concentrations of 1 μ M and 10 μ M. Cellular evaluation of our molecules alone have so shown that in a context without DNA damage, where DNA repair pathways such as homologous recombination are not activated, they have low effect on cell survival. In contrast, for DNA damage response, preliminary results have shown that the molecules entered into the cell and directly acted on RAD51-mediated HR pathway to sensitize DU145 cancer cell to cisplatin treatment. [33]

Table 3: Viability of prostatic cancer cell DU145 in the presence of the selected compounds:



Compound	R ¹	R ²	Cell Viability (% of control)		
			1 μ M	10 μ M	50 μ M
B02	-		95	79	9
8^a (DIDS)	NCS	NCS	90	87	83
12	CN	CN	99	94	100
13	I	I	91	89	84
15	N ₃	N ₃	87	94	93
18	CN	NO ₂	90	85	88
22	PhCO-	PhCO-	100	100	100
23	<i>p</i> OMe-C ₆ H ₄ CO-	<i>p</i> OMe-C ₆ H ₄ CO-	-	-	100
25	<i>p</i> Me-C ₆ H ₄ SO ₂ -	<i>p</i> Me-C ₆ H ₄ SO ₂ -	100	100	100
29	PhOCO-	PhOCO-	100	100	87

^a)commercial compound

In summary, in this work, we have developed analogues of DIDS, a known di-sulfonic stilbene compound identified as a HR potential inhibitor targeting specifically RAD51. Due to suspected chemical or biochemical instability of the isothiocyanate functions of DIDS, we have prepared two series of DIDS analogues bearing small or bulky substituents with various chemical properties, such as cyano, amide, sulfonamide or carbamate analogues. This preliminary structure-activity relationship has highlighted the crucial roles of the sulfonic acid functions as well as the double bond of the stilbene moiety for RAD51 activity. Three compounds, the di-cyano analogue **12**, the di-paramethoxybenzamide **23** and the di-phenylcarbamate analogue **29** with no cytotoxic effect on the selected cell line, have been characterized *in vitro* as potent compounds for inducing RAD51 activity inhibition. This encourage us to go further with *in cellulo* evaluations to ensure that the compounds target RAD51 in this HR pathway and could be proposed for cancer cells sensitization to chemotherapeutic treatment.

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¹ These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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