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

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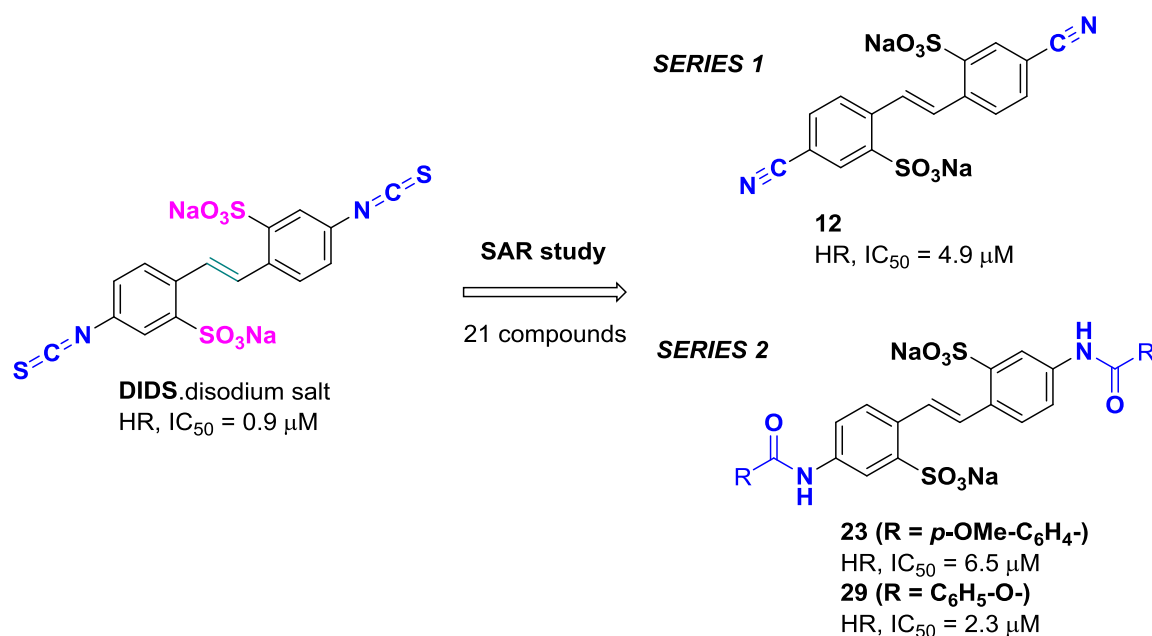
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Abstract

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



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

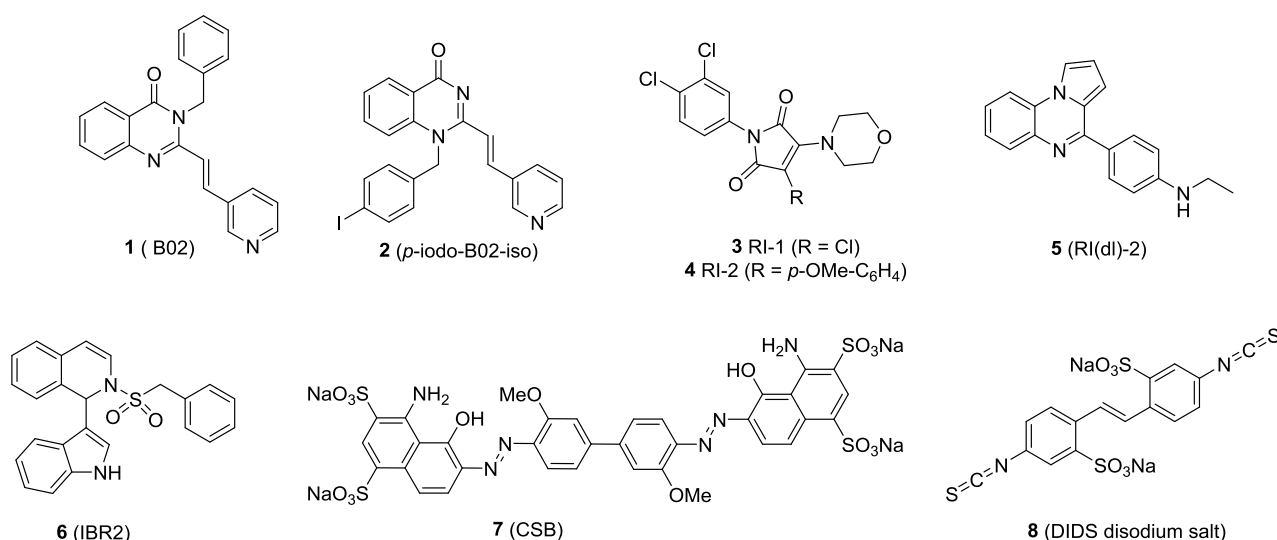
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31 DNA repair process is particularly important in maintaining genomic integrity. In
32 proliferative diseases therapies such as radiotherapy or chemotherapy, this process can restore
33 the DNA damaged with the treatment, and induce a radio- or chemo-resistance.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

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

61 such as artemisinin and chloroquine [13]. With a similar virtual approach Connell and
 62 collaborators identified RI-1 (Figure 1, compound **3**) that disrupted HR with an IC₅₀ of 20
 63 μM. Due to its reactivity as a Michael acceptor [14] inducing an irreversible profile, chemical
 64 optimization of RI-1 was done and substitution of the chloro substituent with a p-
 65 methoxyphenyl group afforded a compound **4** (Figure 1, RI-2) with lower activity but
 66 reversible binding.[15] The same authors applied two parallel screening of more than 8,000
 67 compounds on RAD51-ssDNA binding assay and RAD51-dependent D-loop assay, in order
 68 to identify selective inhibitors. After chemical optimization of a promising lead, compound **5**
 69 (Figure 1, RI (dl)-2) was found to displayed improved D-loop activity, without detectable
 70 effect on RAD51-ssDNA binding.[16] From Nanosyn libraries (24,000 compounds) a
 71 sulfonamide synthetic alkaloid **6** (Figure 1, IBR2) was confirmed by SPR to bind RAD51 and
 72 to inhibit HR by disrupting RAD51 heptamers inducing filament formation.[17] From a
 73 library of 1120 compounds, Chicago Sky Blue (CSB), a dye molecule was revealed as
 74 RAD51 inhibitor in the low nanomolar range (Figure 1, compound **7**).[18] Screening
 75 known biological compounds and drugs, on RAD51 strand-exchange assay *in vitro*,
 76 Kurumizaka's team identified a disulfonic compound 4,4'-diisothiocyanostilbene-2,2'-
 77 disulfonic acid **8** (DIDS), with 50 % effect around 1-2 μM.[19]

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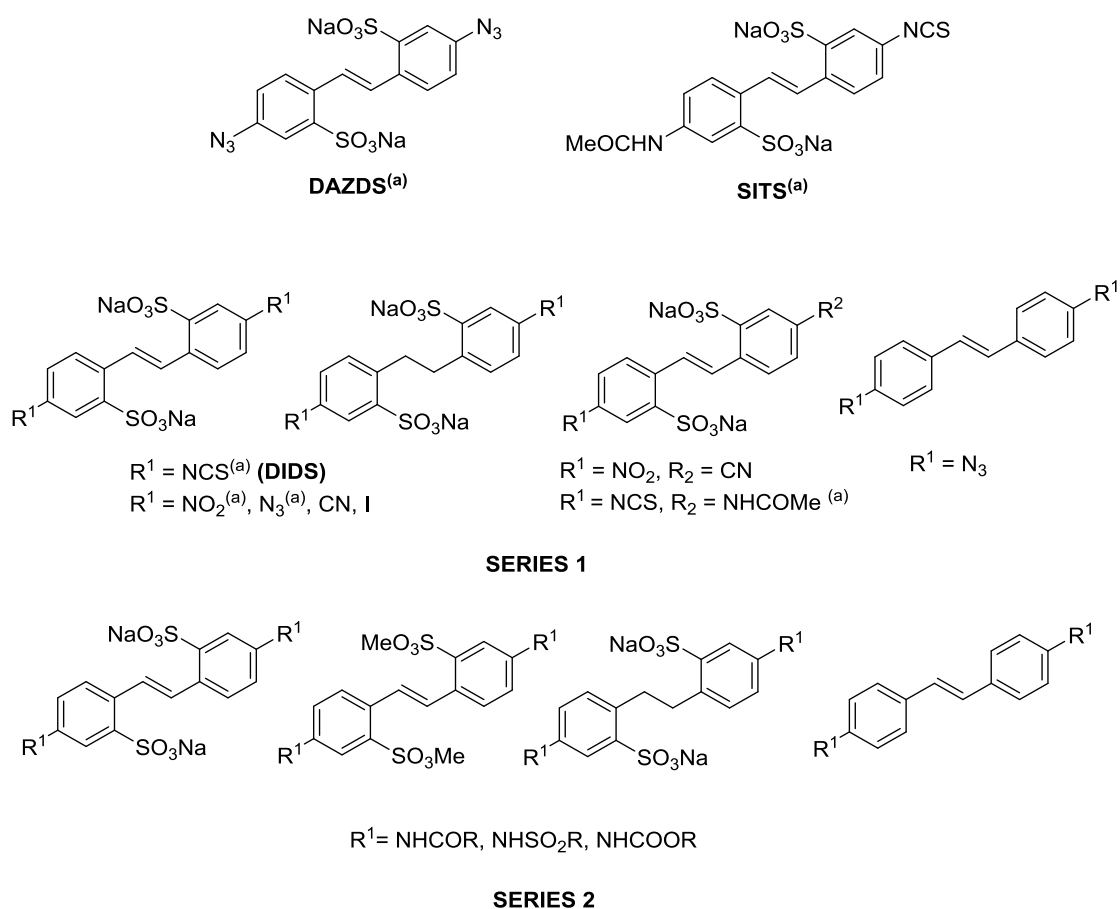
81 **Fig 1.** Small (hetero)aromatic molecules, modulators of the HR process.

82

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

85 suffered from limitations which impair its potential use against cancer, regarding for example
 86 selectivity over other biological targets or chemical stability of the molecule. Especially,
 87 DIDS as inhibitor is not specific of the RAD51 protein. It also inhibits membrane transporters
 88 [20] or anion channels [21], and its isothiocyanate functions may be responsible of some of
 89 these activities. [22] As a chemical weakness, DIDS stability in water has been discussed
 90 [23], and the potential hydrolysis of the isothiocyanate function could afford DIDS oligomers
 91 also identified as chloride channel blockers. [24]

92



93

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

95

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

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

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

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

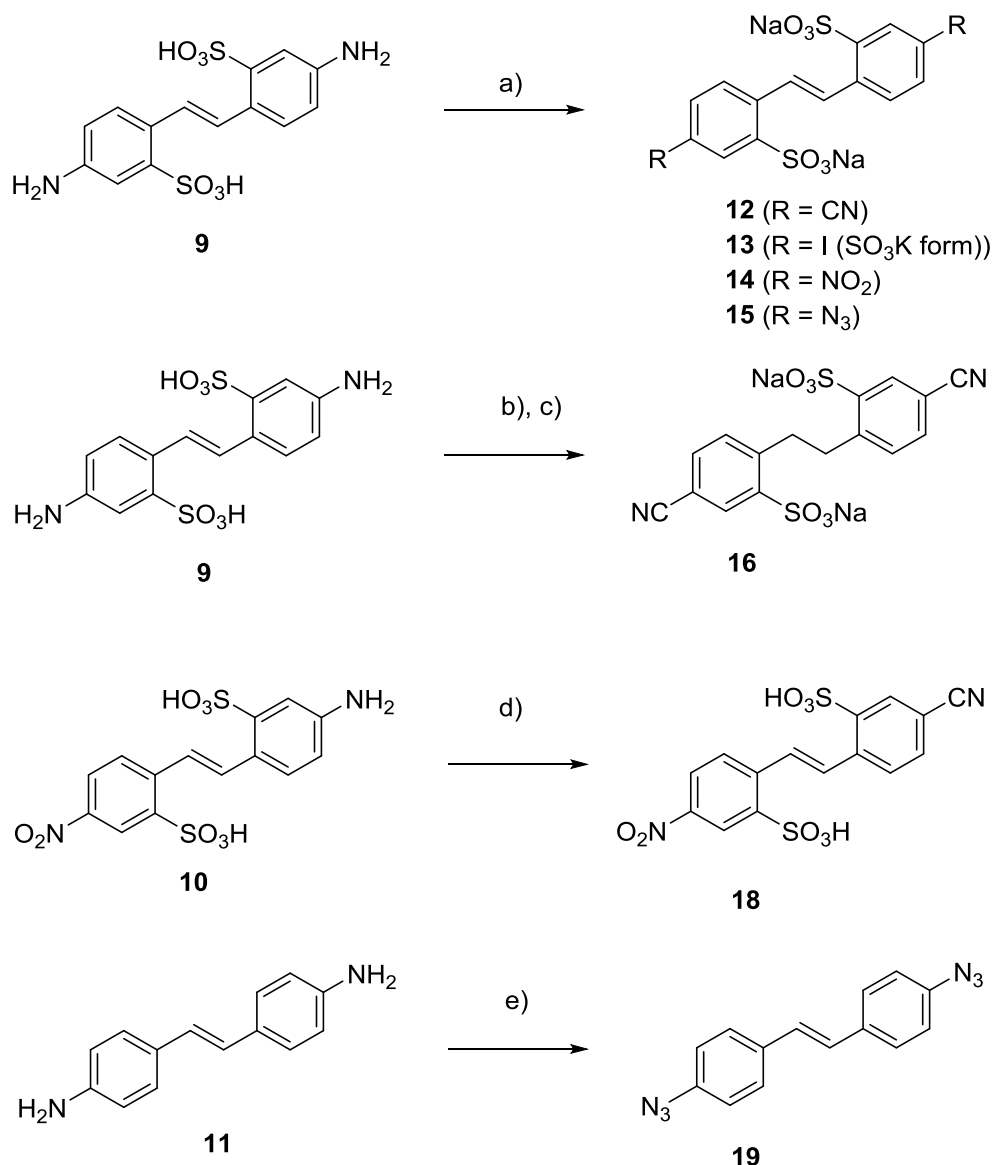
106 Two series of DIDS analogues have been synthesized, the first one including
107 compounds bearing small chemical groups in place of the isothiocyanate group (NCS) in
108 DIDS (Figure 2, series 1) and the second one derived from 4,4'-diamino analogue of DIDS
109 (DADS) to develop compounds such as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

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

126

127

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129

130 **Scheme 1. Synthesis of DIDS analogues of series 1** : Reagents and conditions: a) NaNO₂ (2
 131 equiv), AcOH (5 equiv), NaCN (4 equiv), CuCl (0.25 equiv), H₂O, rt, 2 d, 67% for **12**;
 132 NaNO₂ (2 equiv), AcOH (5 equiv) KI (5 equiv), CuI (0.1 equiv), H₂O, rt, 2 d, 47% for **13**; **14**
 133 and **15** are commercial compounds; b) RaNi, [H₂] 1 atm., NaOH (2 equiv), H₂O, 50 °C, 2 d,
 134 74% of DADB; c) DADB, NaNO₂ (2 equiv), AcOH (5 equiv), NaCN (4 equiv), CuCl (0.25
 135 equiv) water, rt, 3 d, 44% for **16**; d) NaNO₂ (1 equiv), AcOH (3 equiv), NaCN (2 equiv),
 136 CuCl (0.5 equiv), H₂O, rt, 1.5 d, 71%; e) NaNO₂ (2 equiv), AcOH (5 equiv), NaN₃ (2 equiv),
 137 H₂O, rt, 3 h, 90% .
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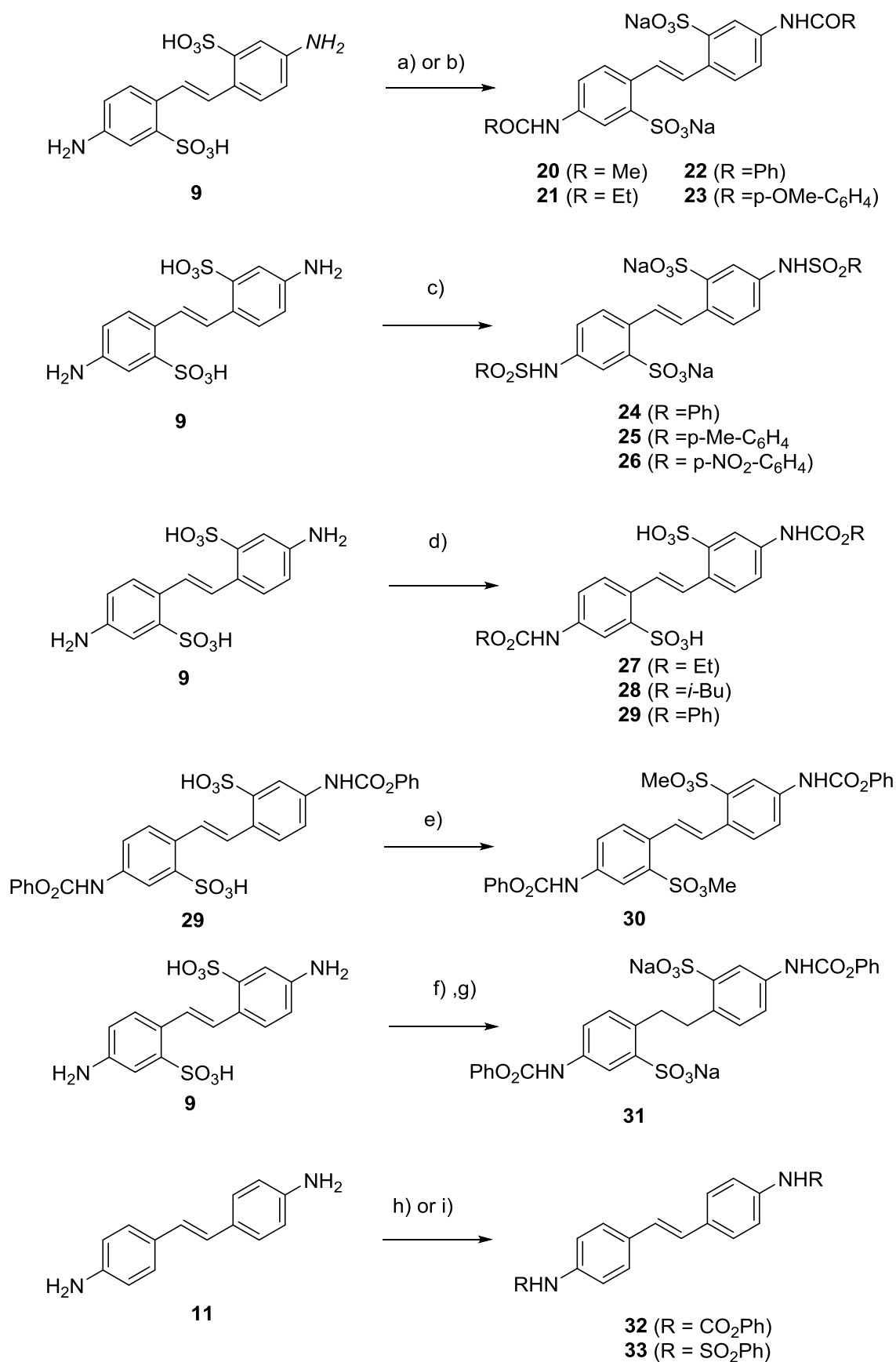
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140 The second series of DIDS analogues, compounds **20-29** and **32-33**, was prepared by
 141 acylation or sulfonylation of the amino precursors **9** or **11** (Scheme 2). In basic aqueous
 142 conditions using anhydrides, sulfonyl chlorides or chloroformates as the reagents, the amides
 143 **20-22**, the sulfonamides **24-26** or the carbamates **27-29** were obtained with good yields (62 to
 144 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

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

155



156

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**

159 (R = Me); overnight, 76 % for **21** (R = Et); overnight, 70 % for **22** (R = C₆H₅); b) RCOCl (4
160 eq.), DIEA (5 eq.), DMF, 80 °C, 4 h, 44 % for **23** (R = p-OMe-C₆H₄); c) RSO₂Cl (2.5 eq.),
161 Na₂CO₃ (5 eq.), H₂O/dioxane (5:1), rt, overnight, 61 % for **24** (R = C₆H₅); 87 % for **25** (R =
162 p-Me-C₆H₄); 81 % for **26** (R = p-NO₂-C₆H₄); d) ROCOCl (2.5 eq.), Na₂CO₃ (5 eq.),
163 H₂O/dioxane (5:1), rt, overnight, 66 % for **27** (R = Et); 67 % for **28** (R = *iso*Bu); 90 % for **29**
164 (R = C₆H₅); e) CH₃C(OMe)₃ (4 eq.), toluene, reflux, 17 h, 32 %; f) Ra-Ni, [H₂] 1 atm., NaOH
165 (2 equiv), H₂O, 50 °C, 2 d, 74% of DADB; g) PhOCOCl (4 eq.), H₂O/dioxane (3:1),
166 overnight, 58 %; h) PhOCOCl (4 eq.), DIEA (6 eq.), CH₂Cl₂, rt, 24 h, 54 % for **32**; i)
167 RSO₂Cl (2.2 eq.), Et₃N (4 eq.), CH₂Cl₂, rt, 24 h, 41 % for **33**.

168
169 With these as-prepared DIDS analogues in hands, we next focused our attention to evaluate
170 them as potential RAD51 inhibitors with two *in vitro* activity tests developed to identify
171 strand exchange activity. The first bioassay is an electrophoresis-based DNA strand exchange
172 [18] between a labeled short single strand ssDNA (58-ssDNA*) and a homologous double
173 strand ds-DNA (33-ds-DNA) catalyzed by RAD51. Using polyacrylamide gel electrophoresis
174 the formation of a novel dsDNA formed (58/33-dsDNA*) could be measured in a dose-
175 dependent manner. The second bioassay is a DNA displacement loop called D-loop [32] using
176 labeled 100-ss-DNA**.

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

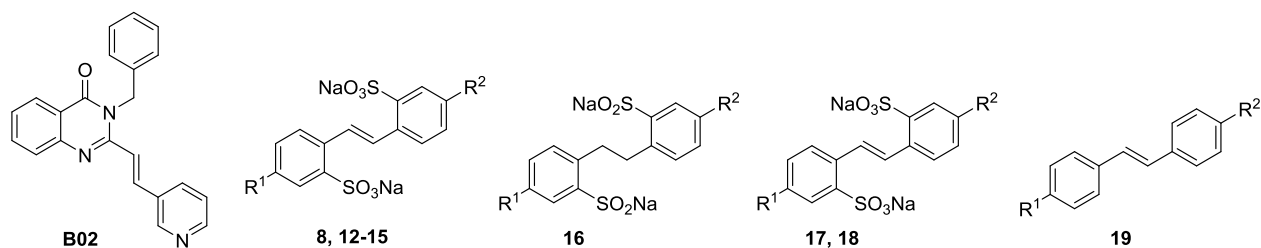
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195 **Table 1** : RAD51 activity evaluation for compounds of series 1

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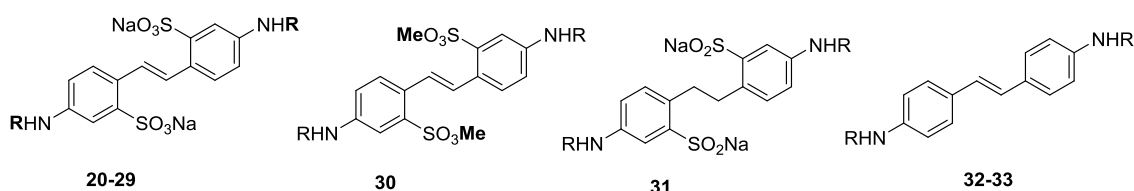


Compound	R ¹	R ²	IC ₅₀ (μM) (DNA strand exchange assay)	IC ₅₀ (μM) (D-loop)
B02	-	-	-	27.4 ^b
8^a (DIDS)	NCS	NCS	0.75	0.9
12	CN	CN	1.3	4.9
13	I	I	17.5	34
14^a	NO ₂	NO ₂	-	>50
15^a (DAZDS)	N ₃	N ₃	10	21.5
16	CN	CN	-	>50
17^a (SITS)	NCS	NHCOMe	-	29
18	CN	NO ₂	-	20
19	N ₃	N ₃	-	>50

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

200 For the second series with bulky amido, sulfonamide or carbonate residues in place of the
 201 NCS one, only the residues with aromatic moieties, such as compounds **23**, **25**, **29**, **31** and **32**,
 202 gave IC₅₀ values in the range of 2.3 μM to 30 μM. Sulfonamide compounds **24-26**, with an
 203 acidic NH proton, were less efficient than amide (compound **23**) or carbonate (compound **29**)
 204 analogues. The best compound in this second series was the diphenylcarbonate **29**, giving
 205 similar results in the electrophoresis-based DNA exchange assay and the D-loop assay, with
 206 IC₅₀ values of 3.8 μM and 2.3 μM respectively. We wanted to block the ionic character of
 207 compound **29**, by esterification of the sulfonic functions. Satisfyingly we observed that the
 208 bis-methyl sulfonate **30** was inactive, pointing out the crucial role of these free sulfonic
 209 functions to block HR activity. Here also, reduction of the double bond to access to
 210 compound **31**, or exclusion of the sulfonic functions (compound **32**) induced a 10-fold
 211 decrease of the activity value, compared to the parent compound **29**.
 212 If the double bond as well as the sulfonic functions of these stilbene compounds appeared as
 213 important elements for RAD51 activity, complementary structure-activity conclusions still
 214 remain difficult. Taking in account that the cyano functions in compound **12** from series 1,
 215 and the benzamide or phenylcarbamate groups of compounds **23** and **29** from series 2, have
 216 no similar physico-chemical properties, this could imply that each series has its specific
 217 interactions in a same binding site, or that each series has its own binding site with RAD51.

218
 219 **Table 2** : RAD51 activity evaluation for compounds of series 2



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221

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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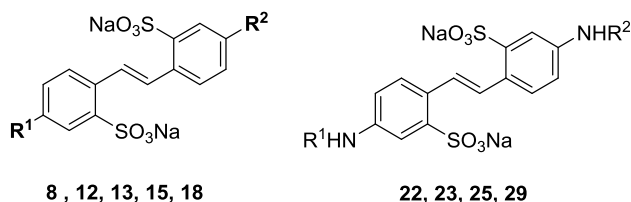
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224 To envisage *in cellulo* evaluation of this family of molecules, the most interesting compounds
 225 were selected and their cytotoxicity was evaluated on prostatic cancer cell (DU145) by the
 226 MTT assay after 72h of exposure at 1μM, 10μM and 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

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

240

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



242

Compound	R ¹	R ²	Cell Viability (% of control)		
			1 μM	10 μM	50 μM
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

243 ^{a)}commercial compound

244

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

258

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263

264 **Notes**

265 The authors declare no competing financial interest.

266

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