IDENTIFICATION AND CHARACTERIZATION OF IN VITRO METABOLITES OF 2-[2'-
(DIMETHYLAMINO)ETHYL]-1,2-DIHYDRO-3H-DIBENZ[DE,H]ISOQUINOLINE-1,3-DIONE
(AZONAFIDE)

CRAIG A. MAYR, SALAH M. SAMI, WILLIAM A. REMERS, AND ROBERT T. DORR

Department of Pharmacology/Toxicology, Department of Pharmaceutical Sciences and Arizona Cancer Center, University of Arizona

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ABSTRACT:

Azonafide (2-[2’-(dimethylamino)ethyl]-1,2-dihydro-3H-dibenz[de,
h]isoquinoline-1,3-dione) is the parent of a new series of anthra-
cene-containing antitumor agents. Its structure is based on
amonafide but lacks a primary amine and has an anthracene chro-
mophore rather than a naphthalene chromophore. Using a rat liver
cytosol incubation and HPLC/MS detection, we have identified four
metabolites resulting from in vitro metabolism of azonafide. These
alkyl-modified derivatives include a mono- and a di-N’-desmethyl
metabolite, an N’-oxide metabolite, and a carboxylic acid metab-
olite. Purified samples of these metabolites were analyzed for
cytotoxic activity using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-
2H-tetrazolium vital dye (mitochondrial reductase) assay and for
inhibition of topoisomerase II (TOPO II) using a cell-free enzymatic
system. Each metabolite had decreased cytotoxicity relative to
azonafide with the following relative potencies in descending or-
der: the mono-N’-desmethyl metabolite, di-N’-desmethyl metabo-
lite, the N’-oxide metabolite, and the carboxylic acid metabolite.
Similarly, the N’-desmethyl metabolites retained TOPO II inhibitory
activity but with lower potency than azonafide. The N’-oxide and
carboxylic acid metabolites did not inhibit TOPO II at 0.05 and 0.5
µg/ml, respectively. Thus, metabolism of azonafide by rat liver
cytosol represents a detoxification pathway rather than a bioacti-
vation scheme for this DNA intercalator.

The azonafide series of anthracene-based antitumor agents was
derived after work by Braña et al. (1980) on a group of agents with a
benzisoquinolinedione-type structure. The new compounds have a
structural resemblance to amonafide (Braña et al., 1980, 1981) but
possess an anthracene chromophore rather than a naphthalene chro-
mophore and lack a primary amine. The structure of azonafide
was derived from work on the cytotoxicity and antitumor activity of
amonafide (Ratain et al., 1991). The parent compound in the
new series is 2-[2’-(dimethylamino)ethyl]-1,2-dihydro-3H-diben-
z[de,h]isoquinoline-1,3-dione and is designated azonafide. It has
shown significant enhancement of antitumor potency as compared to
amonafide when tested against human and murine tumor cells in vitro
and has reduced in vitro cardiotoxicity in rat cells relative to cytotoxic
toxicity in tumor cell lines (Sami et al., 1993, 1995). Prior mechanistic
investigations with azonafide showed that antitumor cytotoxicity
is roughly proportional to DNA intercalation potency, noted by
increased stability against thermal denaturation of double-stranded
calf thymus DNA (Sami et al., 1995). Further mechanistic studies
showed that this agent produces DNA single strand breaks, DNA
double strand breaks, and DNA-protein cross-links characteristic of
TOPO II inhibition, as well as catalytic inhibition of TOPO II activity
(May et al., 1997). The metabolic fate of these active compounds was
not known, nor was the biological activity of any putative metabolites,
a factor that ultimately led to severe toxicity with amonafide in
patients with different metabolizing capabilities.

The current study was designed to characterize the in vitro metab-
olism of azonafide. In this report, we identify four metabolites result-
ing from incubation of azonafide with an S9 fraction of Aroclor-1254
induced rat liver. The metabolites, identified by HPLC/MS, include
two N’-desmethyl species, an N’-oxide metabolite, and a carboxylic
acid derivative of azonafide. The metabolites were screened for
inhibition of DNA-topoisomerase activity using Chinese hamster ovary (CHO) cells and for
cytotoxic activity against Chinese hamster ovary (CHO) cells and for

Send reprint requests to: Robert T. Dorr, The Arizona Cancer Center, P.O.
Box 245024, 1515 N. Campbell Avenue, Tucson, AZ 85724.

1 Abbreviations used are: TOPO II, topoisomerase II; CHO, Chinese hamster
ovary; DMSO, dimethyl sulfoxide; APCI, atmospheric pressure ionization;
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium.

Fig. 1. Structures of amonafide and azonafide.
Structure a is amonafide; structure b is azonafide.
TOPO II inhibition to evaluate the role of metabolism in either bioactivation or inactivation of this series of novel anticancer agents.

Materials and Methods

Drugs. Azonafide analogs were synthesized and purified by chromatography. The structures were confirmed using NMR and compositional analytical techniques as previously described (Sami et al., 1993, 1995). Drugs for cytotoxic analyses were reconstituted into stock solutions of 2 mg/ml (1 mg/ml for the carboxylic acid derivative) in dimethyl sulfoxide (DMSO) (J.T. Baker, analytical grade) and kept at −20°C. Previous studies in this laboratory showed these drugs to be stable when stored in this manner.2

In Vitro Metabolism. In vitro metabolism of azonafide was performed using a modification of the method of Maron and Ames (1983). Azonafide was added to metabolism buffer (35.9 mM KCl, 8.7 mM MgCl₂, 0.11 M KH₂PO₄, 4.0 mM NADP, and 5.4 mM glucose-6-phosphate, pH 7.4) at 20 μg/ml. Incubation mixtures also consisted of Aroclor-1254 induced rat liver S9 fraction (Organon Teknica, Durham, NC) at 1.62 mg protein/ml metabolism mixture. Incubations were carried out at 37°C with agitation for 30, 60, and 120 min. In addition to the incubation samples, samples containing no S9 fraction and samples containing no NADP were incubated for 120 min as negative controls. After incubation, samples were immediately loaded onto C18 Bond Elut columns (Varian, Harbor City, CA) that had been washed three times with methanol and three times with water. The columns were then washed three additional times with water, and the sample was eluted with 1 ml of methanolic 0.1 N HCl. Standards of azonafide and synthesized metabolites were also prepared in metabolism buffer and were extracted on the C18 columns in an identical manner as the samples. Samples and standards were then subjected to HPLC analysis and LC/MS analysis as described below.

HPLC Conditions. Prepared samples (see above) were analyzed for parent drug and putative metabolites by reversed phase HPLC. The basic method used 100 μl of each sample, which was injected onto an Adsorbosphere HS C18, 5 μm, 150 × 4.6-mm column (Alttech, Deerfield, IL) using a Hitachi AS 2000 autosampler (Hitachi, Danbury, CT). Samples were analyzed by using a 4-min isocratic step of 76% 0.02 M ammonium acetate (pH 4.0) vs. acetonitrile followed by an 11-min linear gradient to 65% ammonium acetate using a Perkin Elmer (Norwalk, CT) model 250 biocompatible binary pump system. Ultraviolet absorbance detection was carried out at 265 nm using a Hewlett Packard (Federal Republic of Germany) series 1050 variable wavelength detector, and concurrent fluorescence detection was carried out at 285 nm excitation, 600 nm emission by a BAS FL-45 fluorescence detector (Bioanalytical Systems, West Lafayette, IN). Chromatographic data were collected and processed using the PE Nelson (San Jose, CA) Turbochrome 4 software package. Metabolite peaks were collected and evaporated to dryness in a LABCONCO (Kansas City, MO) Centrivap concentrator under vacuum. Residuals were reconstituted in methanol for MS analyses.

Mass Spectrometry. Collected HPLC peak fractions were subjected to atmospheric pressure chemical ionization (APCI) mass spectrometry, performed on a TSQ tandem mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an APCI source. The APCI source was operated in the negative ion mode, with a vaporizer temperature of 450°C and a corona discharge current of 5 μA. These product scans spanned the mass range of 100 to 500 amu in 3 sec. Product ion spectra of the carboxylic acid metabolite was obtained by subjecting the precursor ion of interest (m/z 305) to collision-induced dissociation with neutral Ar at ~31 eV energy

Cell Culture. Chinese hamster ovary cells (ATCC CCL 61) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in PDRG basal media (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA) and 5% iron-enriched calf serum (Intergen, Purchase, NY). Cells were incubated at 37°C in air with 100% relative humidity. Cells were tested bimonthly and confirmed to be mycoplasma free using a polymerase chain reaction-based mycoplasma detection kit (ATCC).

Microculture Tetratoliumm Assay. This assay is based on reductive cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium (MTT) bромеide to a colored formazan compound as an indicator of cell viability (Alley et al., 1988). Chinese hamster ovary cells were plated at 1000/well onto 96-well microtiter plates (Costar, Cambridge, MA). On day 2, drugs dissolved initially in DMSO and then diluted serially with phosphate-buffered saline (pH 7.4) were added at concentrations of 1 nM to 10 μM in half-log gradations. Final concentrations of DMSO did not exceed 0.1% (v/v). Drugs were removed after 1 hr of incubation; the plates were then washed twice with phosphate-buffered saline and incubated with drug-free media at 37°C in air with 100% relative humidity for 5 days. After the 5-day incubation period, 50 μl of a 1 mg/ml MTT solution were added to each of the wells, and the plates were incubated an additional 4 hr. The medium was then aspirated, and the formazan product was solubilized by DMSO (100 μl/well). The intensity of the color, which is proportional to viable cell numbers, was quantified by absorbance at 540 nm on an automated microculture plate reader (Biotek Instruments). Test results were calibrated in per cent control absorbance from untreated CHO cells. Each drug concentration was tested in three independent experiments, and the IC₅₀ values were determined using the dose response analysis function (sigmoid fit) in the data graphing software package Microcal Origin (version 4.10, Microcal Software Inc., Northampton, MA).

Topoisomerase II Inhibition Assay. Drug-induced TOPO II inhibition was assessed using the Topoisomerase II Drug Screening Kit from TopoGEN Inc (Columbus, OH), which detects any covalent links between TOPO II and DNA (the “cleavable complex” reviewed by Ross, 1985). Briefly, 0.5 μg of pRYG plasmid, which contains a single high affinity TOPO II recognition and cleavage site, was incubated with increasing concentrations of parent or metabolites (0.05, 0.5, and 5.0 μg/ml) plus purified human TOPO II (6 units). Incubations were carried out at 37°C for 30 min in cleavage buffer (30 mM Tris-HCl, pH 7.6, 3.0 mM ATP, 15 mM mercaptoethanol, 8.0 mM MgCl₂, 60 mM NaCl). The reaction was stopped, and cleavable complex trapping was accomplished by the addition of 1/10 volume of 10% SDS. Proteinase K was then added (50 μg/ml), and the samples were incubated at 37°C for 15 min. One-tenth volume of loading dye (0.25% bromphenol blue, 50% glycerol) was then added, and samples were extracted twice with chloroform:isoamyl alcohol (24:1). Samples were electrophoresed on a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide at 45 V overnight in 4× TAE buffer (Foglesong and Reckford, 1992) also containing 0.5 μg/ml ethidium bromide. Gels were visualized on an Eagle Eye II video imaging system (Stratagene) using the negative image function in the Stratagene Eagle Sight software package, version 3.1.
Identification of Metabolites. The *in vitro* metabolism of azonafide was carried out according to a modification of the method of Maron and Ames (1983) with Aroclor-1254 induced rat liver S9. The HPLC analysis of the S9 incubation samples produced chromatograms with five distinct peaks at each time point (fig. 2). The peaks were collected and subjected to MS analyses, which produced five molecular ions of *m/z* 290, 304, 318, 334 and 305 (table 1). These molecular ions correspond to the molecular weight of the compounds, as negative chemical ionization mode was employed. The assignment of chemical structures was based on the molecular weights of the metabolites and, for peak 5, the collision-induced dissociation fragmentation spectra (table 1). Furthermore, the synthetic standards of the metabolites coeluted with the metabolite peaks in the HPLC analyses (fig. 2). The mass spectra of the metabolites were confirmed by MS analysis of synthetic standards (table 1). Peaks 1 and 2 were identified as mono- and di-*N*-desmethyl metabolites of azonafide, respectively. Peak 3 was determined to be the parent compound, peak 4 was identified as a *N*-oxide metabolite, and peak 5 was identified as a carboxylic acid derivative of azonafide (fig. 3).

Quantitation of the metabolites present at the 120-min time point revealed the recovery of 1.15 ± 0.3 μg/ml for the di-*N*-desmethyl metabolite, 3.67 ± 0.4 μg/ml for the mono-*N*-desmethyl metabolite, 0.44 ± 0.3 μg/ml for azonafide, 1.50 ± 0.9 μg/ml for the *N*-oxide metabolite, and 8.46 ± 0.7 μg/ml for the carboxylic acid metabolite. This recovery accounts for 78.9 ± 14.2% of the original azonafide in the samples. The NADPH-free control yielded a recovery of 16.74 ± 0.6 μg/ml of unchanged azonafide from the original 20 μg/ml sample, representing a recovery of 83.7 ± 3.1%.

Cytotoxicity. The growth inhibitory potencies of the parent and four metabolites toward CHO cells were assessed using an MTT vital dye assay. Table 2 shows the results of these experiments. The parent compound had an *IC*<sub>50</sub> of 68.2 nM in these assays. The *IC*<sub>50</sub> values for the mono- and di-*N*-desmethyl metabolites were 265 and 981 nM, respectively. The *N*-oxide and carboxylic acid metabolites had no cytotoxic activity at concentrations up to 10 μM, the highest tested in these assays. At these concentrations tested, the *N*-oxide and carboxylic acid metabolites reduced cell viability to only 61.8 ± 6.7% and 97.1 ± 4.6% relative to control, respectively.

Topoisomerase II Inhibition. Drug-induced TOPO II inhibition was assessed using a commercial topoisomerase II drug screening kit in which supercoiled pRYG plasmid DNA, containing the high affinity TOPO II cleavage and recognition site, was incubated with increasing concentrations of parent drug or purified metabolites (0.05, 0.5, and 5.0 μg/ml) plus purified human TOPO II. Fig. 4 shows a typical gel obtained from these experiments. The parent compound (lanes 5–7) inhibited the TOPO II-dependent relaxation of pRYG plasmid with increasing concentrations. The two *N*-desmethyl metabolites also inhibited TOPO II activity (lanes 8–10) for the di-*N*-desmethyl metabolite and lanes 11–13 for the mono-*N*-desmethyl metabolite) but with increasing concentrations required (i.e. decreased inhibitory potency). This is noted by the inhibition of TOPO II dependent relaxation of the pRYG plasmid. The *N*-oxide metabolite did not show any inhibition of TOPO II activity at 0.05 and 0.5 μg/ml (lanes 14 and 15). However, the migration of the pRYG plasmid in the gel was only altered by 5.0 μg/ml of the *N*-oxide metabolite in the absence of TOPO II (not shown). This makes the interpretation of TOPO II inhibition by the *N*-oxide at this concentration impossible (lane 16). The carboxylic acid derivative showed no TOPO II inhibition at any concentration tested (lanes 17–19).

Discussion

We have previously reported that azonafide and its analogs are potent cytotoxic compounds in several human and rodent tumor cell lines, both *in vitro* and *in vivo* (Mayr et al., 1997; Remers et al., 1994; Sami et al., 1993). The azonafides act as DNA intercalators, where cytotoxic potency is roughly equivalent to DNA binding strength, as assessed by DNA thermal melt transitions (Sami et al., 1995). These agents inhibit DNA and RNA synthesis and produce DNA single and double strand breaks in CHO cells. Furthermore, we have investigated

![FIG. 3. Structures of identified metabolites of azonafide.](https://example.com/fig3)

*Structure a* is the di-*N*-desmethyl metabolite (peak 1, mol wt 290), *structure b* is the mono-*N*-desmethyl metabolite (peak 2, mol wt 304), *structure c* is the *N*-oxide metabolite (peak 4, mol wt 334), and *structure d* is the carboxylic acid metabolite (peak 5, mol wt 305).

![TABLE 1](https://example.com/table1)

<table>
<thead>
<tr>
<th>Peak</th>
<th><em>m/z</em></th>
<th>Metabolite Assignment</th>
<th><em>m/z</em> of Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>290</td>
<td>Di-<em>N</em>-desmethyl</td>
<td>290</td>
</tr>
<tr>
<td>2</td>
<td>304</td>
<td>Mono-<em>N</em>-desmethyl</td>
<td>304</td>
</tr>
<tr>
<td>3</td>
<td>318</td>
<td><em>N</em>-Oxide</td>
<td>318</td>
</tr>
<tr>
<td>4</td>
<td>334</td>
<td>318, 304, 273</td>
<td>334, 318, 273</td>
</tr>
<tr>
<td>5</td>
<td>305</td>
<td>Carboxylic acid</td>
<td>305, 305, 246</td>
</tr>
</tbody>
</table>

* MS/MS analysis was used in the identification of peak 5.
* See fig. 2.
* Mass-to-charge ratio for the ionized parent and fragment ions.

![TABLE 2](https://example.com/table2)

<table>
<thead>
<tr>
<th>Agent</th>
<th><em>IC</em>&lt;sub&gt;50&lt;/sub&gt; (nM) [SD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azonafide</td>
<td>68.2 [5]</td>
</tr>
<tr>
<td>Mono-<em>N</em>-desmethyl</td>
<td>265 [20]</td>
</tr>
<tr>
<td>Di-<em>N</em>-desmethyl</td>
<td>981 [27]</td>
</tr>
<tr>
<td><em>N</em>-Oxide metabolite</td>
<td>NA *</td>
</tr>
<tr>
<td>Carboxylic acid metabolite</td>
<td>NA *</td>
</tr>
</tbody>
</table>

* Continuous drug exposure time was 1 hr in the MTT assays; total incubation time in assays was 5 days. Results are based on three independent experiments for each agent.
* Not active.
the catalytic inhibition of topoisomerase II by these compounds in cell-free enzymatic systems (Mayr et al., 1997).

In the present study, we have identified and characterized the activity of four major metabolites of azonafide formed by rat hepatic enzymes in vitro. Two metabolites identified in this study, the mono-N'-desmethyl species and the N'-oxide metabolite, are consistent with those reported for amonafide in both humans and dogs. Felder et al. (1987) identified eight metabolites of amonafide in human urine and plasma after a 30-min infusion of 400 mg/m². These included an N'-oxide metabolite, an N-acetyl-N'-oxide metabolite, and an N-acetyl-N'-desmethyl metabolite. In dogs receiving 100 mg of amonafide/m², Lu et al. (1988) identified an N'-oxide metabolite in plasma, urine, and bile and a mono-N'-desmethyl metabolite in urine and bile only. This is of interest relative to the current study as dogs have an insufficient ability to acetylate aromatic primary amines. In humans, amonafide produced severe myelosuppression in patients with a rapid acetylator phenotype (Ratain et al., 1991). This discouraged further clinical development even though therapeutic activity was observed in patients with solid tumors (Costanza et al., 1995).

Azonafide contains no aromatic primary amine moiety and thus is not a substrate for acetylation.

The identification of a di-N'-desmethyl metabolite and a carboxylic acid-containing metabolite was not reported for amonafide and seems to be unique to azonafide. A conceivable pathway for formation of the carboxylic acid metabolite involves deamination of the tertiary amine of azonafide and/or the secondary or primary amines generated by formation of the N'-desmethyl metabolites. These reactions would yield an aldehyde intermediate that may be unstable and rapidly oxidized to the carboxylic acid. Of interest, the structurally similar anthracene-based intercalator, mitoxantrone, was reported metabolized to both monocarboxylic acid and dicarboxylic acid metabolites (Blanz et al., 1991; Chiccarelli et al., 1986; Ehninger et al., 1985). However, these metabolites are produced by the oxidation of primary alcohol moieties on the 1,4-substituted hydroxyethylamino side chains in mitoxantrone and not by the presumed deamination/aldehyde oxidation of the aminoethyl side chain of azonafide or the N'-desmethyl metabolites.

Our cytotoxicity analyses of the azonafide metabolites indicate that in vitro metabolism of azonafide represents a detoxification pathway rather than a bioactivation scheme. The metabolites were either less active than azonafide or completely inactive against CHO cells in MTT vital dye assays. Sequential demethylation of the (dimethyl)aminoethyl side chain of azonafide resulted in decreased cytotoxicity. Each demethylation event resulted in about a half-log decrease in cytotoxic potency (table 1). Previous studies in our lab with these demethylated azonafide analogs have shown similar results (Remers et al., 1994). In a screen of four tumor cell lines, normal and resistant L-1210 mouse leukemia cells, OVCAR-3 human ovarian carcinoma cells, and A-375 human malignant melanoma cells, azonafide and the mono-N'-desmethyl metabolite had approximately equal mean cytotoxic potencies. The di-N'-desmethyl analog was approximately 4-fold less potent in these experiments. Though the isolation of N'-desmethyl metabolites of amonafide has been published (Felder et al., 1987; Lu et al., 1988), we know of no report describing the cytotoxic activity of these species. The lack of cytotoxicity of the N'-oxide metabolite of azonafide is not surprising in light of previous studies of amonafide metabolites. Felder et al. (1987) tested N-acetyl- and N-oxide metabolites of amonafide for in vitro activity against P388 murine leukemia cells. This group showed that the (N(5))-acetyl metabolite had only slightly less activity than amonafide but that the N'(N(1))-oxide metabolite was inactive against this tumor cell line. Thus, the carboxylic acid metabolite of azonafide was inactive in the cytotoxicity analyses and is not surprising based on the activity of other synthetic azonafide analogs. Of 140 analogs of azonafide synthesized, those lacking an amine function in the side chain at the N2 position had decreased cytotoxic potencies when compared with azonafide (Remers et al., 1994). The magnitude of these decreases in potency ranged from 5- to 350-fold.

The potency for inhibition of TOPO II by these metabolites is also consistent with their cytotoxic potencies. A previous study has shown that azonafide, and selected analogs, inhibit the catalytic activity of TOPO II in a cell-free enzymatic system (Mayr et al., 1997). This prior report also demonstrated that these agents do not alter the migration of the pRYG plasmid during agarose gel electrophoresis. In the current study, we show that the N'-desmethyl metabolites retain TOPO II inhibitory activity but with decreased potency. Similar to the cytotoxicity results, TOPO II inhibitory potency is decreased by demethylation at the (dimethylamino)ethyl side chain of azonafide, and the nontoxic metabolites did not inhibit TOPO II activity. Interestingly, the N'-oxide metabolite alters the mobility of the pRYG plasmid in agarose gel electrophoresis at high concentrations. This phenomenon has not been observed for any azonafide analog tested in this system and may be a result of altered electrostatic interactions with DNA. Unfortunately, it renders impossible the interpretation of TOPO II inhibition by this metabolite at the highest concentration tested.

The results of this study demonstrate the detoxification of azonafide by in vitro metabolism. It is interesting to note that the activity of azonafide, including cytotoxicity and TOPO II inhibition, is diminished going from a tertiary amine to a secondary or primary amine in the (dimethylamino)ethyl side chain. Activity of azonafide is abolished by deamination and subsequent oxidation to a carboxylic acid group in the side chain, demonstrating the necessity of an amine moiety in this position for activity. Additionally, perturbation of the azonafide tertiary amine, i.e., the generation of an N-oxide, results in the loss of both cytotoxic activity and TOPO II inhibition. This reinforces the requirement for a tertiary amine at the side chain position for optimal antitumor activity.
Overall, the *in vitro* metabolism of azonafide by rat liver cytosol results in metabolites with decreased cytotoxicity and decreased potency of TOPO II inhibition. This finding is significant in light of the life-threatening myelosuppression associated with the metabolism of amonafide in some cancer patients. Based on this *in vitro* analysis, it seems that azonafide, and perhaps its analogs, may be free of the unpredictable side effects that prevented the routine clinical use of amonafide.

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**References**


