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Hepatitis C Viral IRES Inhibition by Phenazine and Phenazine-Like Molecules

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Abstract—An in vitro assay based on the expression of Fluci reporter gene under the translational control of HCV IRES was used to evaluate and screen compound libraries. A structure–activity relationship study on a phenazine hit was conducted. Our data suggest that an intact phenazine or phenazine-like core with two distal polar substitutions is crucial for potency. © 2000 Elsevier Science Ltd. All rights reserved.

It is estimated that approximately 3% of the world's population is infected with hepatitis C virus (HCV), a member of the Flaviviridae. As HCV is a major cause of post-transfusion and community acquired non-A non-B hepatitis, it poses a great threat to the public health. The acute symptoms of this infection include jaundice and fatigue. More importantly, this infection persists in >80% of the infected population. The chronic infection often leads to liver cirrhosis which is associated with a high risk of the development of hepatocellular carcinoma.[1]

Much effort is being directed towards the understanding of HCV replication and the disease associated with it. In particular, research has been focused on the molecular biology of viral protein functions.[2] It has been shown that the non-structural protein NS3, which requires NS4A cofactor[3c,d] for enhanced activity, encodes protease functions for polyprotein processing hence is likely to play a key role for downstream regulation of viral replication.[3,4] One of such protein products, (i.e., NS5B), is found to encode functions as RNA-dependent-RNA polymerase responsible for viral RNA replication.[5] Alternatively, HCV RNA genome has been the subject for the investigation. Studies[6] show that HCV internal ribosome entry site (IRES), the 'landing pad' contained within the genomic 5′ non-coding region, is required for translation initiation. The unique viral secondary RNA structure would offer a potentially valid target for early intervention in the viral life cycle. Several fluorenones and xanthones have shown inhibitory properties in vitro against HCV IRES-dependent translation.[7] For a broader search of low molecular weight anti-IRES agents, we have adopted a strategy which allows quick hit identification, validation and subsequent optimization. A library of 132,000 compounds and fungal extracts were screened using an in vitro assay[8] in which a molecular construct (CAT-IRES-Fluci) was expressed in Huh7 cells under a CMV promoter. The HC40 cell line Luciferase activity would therefore report IRES-dependent translation. Compounds showing inhibition in this assay were further evaluated in cell toxicity assay[9] to establish the toxicity profile of the inhibitors. As a result of such screenings, several chemical classes emerged, showing activity in Luciferase assay with only low level of toxicity. In this communication, we describe our investigations on phenazine series as novel anti IRES agents. The hit molecule **1** (also known as neutral red) exhibited potent anti-IRES activity which was confirmed by repeated assays. Chemically, the planar structure stretched to allow extended nitrogen–arene conjugation and, as a result, was highly colored. The two distal amino substitutions together with the ring nitrogen atoms could potentially form a unique hydrogen bonding array. In view of the strong staining effect as an undesired feature with compound **1**, a structural modification became necessary.

In order to evaluate the necessity of the ring system defined by structure **1** for IRES activity, a number of molecules lacking a central ring were tested. The results

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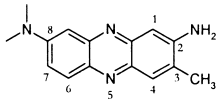
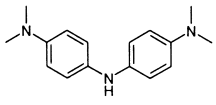
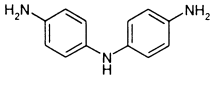
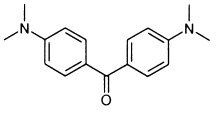
of some representative examples are summarized in Table 1. The crucial role played by the central ring was evident, as all 'open' analogues (**2**–**4**) displayed lower, or no activity relative to the parent molecule. It was conceivable that the angle formed by the central linking group (NH or CO) and two flanking aryls was now widened.[10] The observation that **2** retained residual activity appeared to also indicate the importance of both alkylated amine and bridging nitrogen, as **3** or **4** lacking either one of these features were completely inactive.

Subsequently, our investigation was focused on intact phenazine or phenazine-like cores with structural changes limited to only ring size and substitutions. We expected that the modified ring structures would be more amenable to chemical manipulations needed for achieving optimal activity.

Among modifications of ring substituents, the 7-amino function was chosen initially for replacement by a halogen function via Sandmeyer reaction (Scheme 1). This step should provide analogue(s) lacking one amino function for direct comparison with the parent structure. Chemically, the halogen function could be used as a handle to introduce a variety of groups. The standard Sandmeyer reaction carried out in aqueous medium at 0 °C afforded formation of diazonium ion which gave, upon treatment with potassium iodide, compound **5** as the only product.[11]

Synthesis of a novel ring system was exemplified by versatile, drug-like dibenzoxazapinones. Upon condensation of *o*-hydroxyanilines with *o*-chloroaryl acids,[12] amides **10**–**11** were obtained. Cyclization, followed by catalytic hydrogenolysis, afforded diamino-benzoxazepinone **14** as well as monoamino-benzoxazepinones **15** in good yields.[12]

Table 1. Anti-IRES activity of compounds with truncated central ring

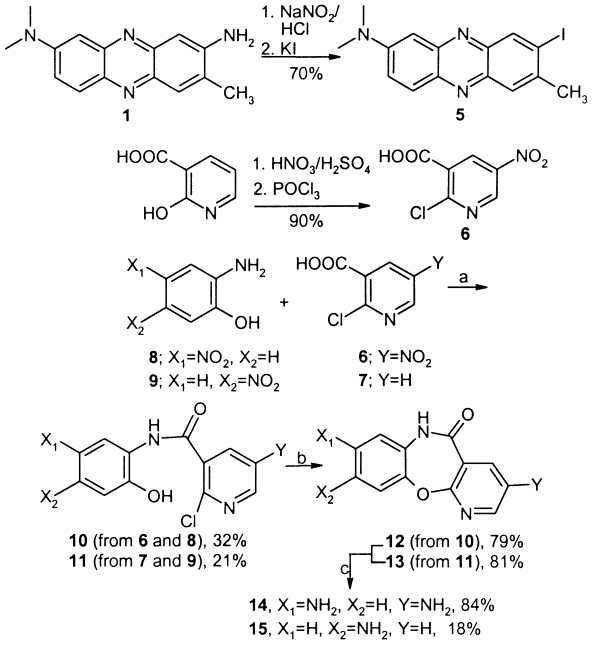
Compounds	Structures	Activity Fluci (HC40) IC_{50} (μg/mL)
1		0.02
2		2.19
3		>10
4		>10

Other compounds listed in Table 2 were all acquired from commercial sources. Chemical integrity of all analogues was secured by ^{1}H NMR, mass spectroscopy (for synthetic analogues) and HPLC analysis prior to biological evaluations.[11]

As shown in Table 2, the anti-IRES activity was very sensitive to structural variations. Phenazines **16**–**20**, while maintaining same or similar core structure like **1**, all displayed reduced activity. It was apparent that the concurrent involvement of multiple functions in **1** in the binding was mandatory for good activity. For example, merely changing dimethylamine function to a hydroxyl group as seen in **16** resulted in a significant drop in activity. Similarly the replacement of amine function in **1** with iodide abolished the activity. On the other hand, the activity was affected to a lesser degree with arylation of the central nitrogen (as seen in **18**). All phenazine analogues showed respectable toxicity margin in MTT assay in HC 40 cell lines.[9]

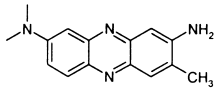
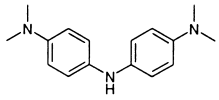
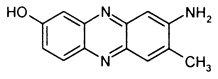
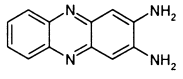
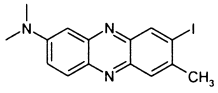
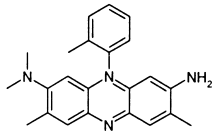
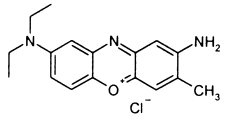
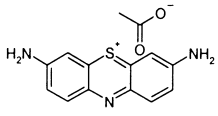
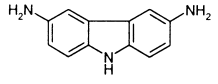
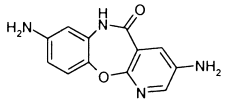
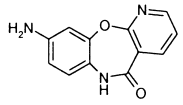
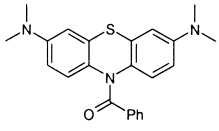
Interestingly, phenazine-like compounds **19** and **20** carrying permanent charges were comparatively active, although notably more toxic than their phenazine counterparts. The attempt to adjust the ring size by using structurally similar carbazole and benzoxazepinone templates (**14**, **15** and **21**) resulted in complete loss of activity. The interruption of conjugation rather than size change appeared to be one of the causes as compound **22** having standard 6-6-6 ring arrangement but lacking the conjugation was, too, inactive.

Although the measurement of Fluci signal in HC40 cells largely reflected the level of IRES activity, non specific transcriptional and translational modulation could not be excluded. A CMV-Fluci assay in HCF cells (pooled Huh-7 cells stably transfected with CMV-Fluci) was



Scheme 1. (a) $SOCl_2$/THF/DIPEA; (b) from **11**, pyridine, 90 °C; from **12** and **13**, NaOH/DMF; (c) H_2/Pd.

Table 2. Anti-IRES activity of phenazine and phenazine-like analogues

Compound	Structure	Fluci IC_{50} (HC40) (μg/mL)	IC_{50} (HCF)/IC_{50} (HC40)	MTT (HC40) CC_{50} (μg/mL)
1		0.02	10	>10
2		2.19	0.8	2.3
16		3.74	>2.7	>10
1		4.03	2.5	>10
5		>10	—	>10
18		~1	1	>10
19		1.00	3	<1
20		2.86	1.7	4.67
21		>10	—	>10
14		≫10	—	>10
15		≫10	—	≫10
22		≫10	—	≫10

therefore employed as a secondary assay. In this system the measured Fluci signal would still be under the CMV transcriptional promoter control but not under IRES translational control. As seen in Table 2, there appeared some level of separation in the two Fluci assays for several compounds, with the ratio of HC40 versus HCF in the rank of **1** (10) > **16**, **17**, **19** (2–3) > **18**, **20** (1–<1).

As a crucial RNA genomic structure required by virus for initiation of translation, HCV IRES would appear to provide a unique target for pharmaceutical intervention. Our dual luci testing system has served effectively for the selection of promising compounds for further downstream studies. In the absence of suitable reference compounds, multiple assays are often needed in order to reach reliable conclusions. The additional evaluation in vivo[13] on compound **1** appeared to lend support for the validity of the dual luci assay strategy.

In conclusion, our search for inhibitors of HCV IRES target has led to the identification of a novel phenazine series. The preliminary SAR study has established that the phenazine core and polar substituents such as amines at positions 2 and 8 are crucial for potent activity, possibly due to the possession of unique hydrogen bonding array. The reduced activity observed with the 'open' analogues **2**–**4** were indicative of change of the array as a result of, at least partially, geometry changes. The results also suggested that a pyrazine moiety is important but not essential for activity as phenazine-like molecules (**19** and **20**) lacking a pyrazine arrangement were also comparatively active although more toxic. While alteration of **1** has resulted in drop in activity, good toxicity profile and moderate selectivity retained in **16** and **17** suggest that there is room for further improvement of the structures.

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8. (a) Wang, C.; Sarnow, P.; Siddiqui, A. *J. Virol.* **1994**, *68*, 7301. (b) Fluci assays in HC 40 and HCF cells: the HC 40 and HCF cell lines were derived from hepatocellular carcinoma cell line Huh-7. The cell line was transfected with the dicistronic construct pCMV-CAT-IRES-Fluci for HC 40 and with pCMV-Fluci construct for HCF. The following conditions were used for both assays: 24 h prior to the assay, cells were seeded at 1.5×10^4 cells/well in a 96-well plate. The compounds were then added at various concentrations in a final DMSO concentration of 0.2% and incubate 24 h at 37 °C. Following the incubation the cells were washed twice in PBS. 50 μL of Luciferin lysis buffer is added and kept for 10 min in the dark. Cells were then re-suspended and 80 μL was taken on a white 96-well plate for Luciferase activity.
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10. A molecular mechanics calculation using HyperChem program suggests, in addition to the widened angle, a slight pyramidal configuration for the central nitrogen atom in structures **2** and **3**.
11. The synthetic analogues have been characterized by the NMR analysis (spectra recorded on Varian 300 MHz or Bruker 400 MHz spectrometer), MS analysis (using a Finnigan MAT LCQ system) and HPLC analysis (using 0.01% TFA buffered water/acetonitrile system on a reverse phase C18 VYDAC column with UV detector set at 215 nm wave length). For compound **5**, ^{1}H NMR ($CDCl_3$): δ, 2.68 (s, 3H), 3.22 (s, 6H), 7.04 (d, $J=2.7$ Hz, 1H), 7.60 (dd, $J_1=9.5$ Hz, $J_2=3$ Hz, 1H), 7.96 (s, 1H), 7.97 (d, $J=9.5$ Hz, 1H), 8.68 (s, 1H); MS^+, 164.2; HPLC purity: >87%. For compound **14**, ^{1}H NMR (DMSO): 10.26 (s, 1H), 7.65 (d, $J=3.0$ Hz, 1H), 7.31 (d, $J=3.0$ Hz, 1H), 6.84 (d, $J=8.5$ Hz, 1H), 6.25–6.20 (m, 2H), 5.36 (s, 2H), 5.09 (s, 2H), MH^+: 242. For compound **15**, ^{1}H NMR (DMSO): 10.27 (s, 1H), 8.41 (dd, $J=5.0$ Hz, $J=2.0$ Hz, 1H), 8.17 (dd, $J=7.5$ Hz, $J=2.0$ Hz, 1H), 7.38 (dd, $J=7.5$ Hz, $J=4.5$ Hz, 1H), 6.78 (d, $J=8.5$ Hz, 1H), 6.47 (d, $J=2.5$ Hz, 1H), 6.35 (dd, $J=8.5$ Hz, $J=2.5$ Hz, 1H), 5.20 (s, 2H); MH^+: 227. All commercial compounds showed purity >90% by HPLC analysis.
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